MECHANISM OF ACTION OF NATURALLY OCCURRING ANTIOXIDANTS
(Mechanism of action of Uric Acid and Capsaicin)

THESIS

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SAURABH SINGH

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF LIFE SCIENCES
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)

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Dedicated

to the memory of

my Grandfather

Shri Girish Chandra Pant

TELEPHONE: 400741
TELEX: 564-230 AMU IN

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF LIFE SCIENCES
ALIGARH MUSLIM UNIVERSITY
ALIGARH--202 002 INDIA

REF. NO.....

DATED 9.3.2000

CERTIFICATE

I certify that the work presented in this thesis has been carried out by **Mr. Saurabh Singh** under my supervision. It is original in nature and has not been submitted for any other degree.

(S.M.Hadi)

Professor and Chairman

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Summary

It has been proposed that considerable DNA damage may be caused by endogenous metabolites produced during the body's normal metabolic processes. Such reactions are considered to lead to the formation of oxidised bases, alkylated bases and exocyclic bases. Evidence has indicated that exocyclic base adducts exist at significant levels in hepatic DNA of rats and humans and in *E.coli* these are efficient premutagenic lesions. It is considered that such endogenous DNA damages may contribute to the etiology of genetic diseases and may define the baseline of human cancer. Previous studies in this laboratory have shown that L-DOPA causes oxidative DNA damage and that the reaction is catalysed by the formation of hydroxyl radicals. Uric acid is considered to be a naturally occurring antioxidant and is present in the plasma at a relatively higher concentration. In order to further explore the antioxidant functions of uric acid, I have investigated its effect on L-DOPA- Cu (II) mediated DNA cleavage at concentrations similar to or lower than those found in plasma. These studies constitute the first chapter of the results.

I have also compared the antioxidant role of uric acid with its structural analogue and metabolite xanthine with respect to *in vitro* DNA damage by L-DOPA and Cu(II). Results indicate that uric acid is able to significantly reduce the extent of DNA damage by L-DOPA-Cu(II). Also, compared to uric acid, xanthine is a better scavenger of hydroxyl radical. However, both uric acid and xanthine inhibited the DNA damage to a greater extent than some of the known scavengers of ROS such as mannitol and thiourea.

The food that we eat has a significant bearing on our overall health. Previous studies in this laboratory have confirmed that several classes of plant derived polyphenolic antioxidant compounds such as flavonoids (Said Ahmad *et al.*, 1992; Fazal *et al.*, 1990) tannins (Bhat and Hadi, 1994; Khan and Hadi, 1994) and stilbenes are themselves capable of oxidative DNA damage in the presence of transitional metal ions. Capsaicin or trans-8-methyl-N-vanillyl-6-noneamide is the principal pungent phenolic substance present in hot red and chili peppers, which are an important part of various diets around the world. As described above, there are numerous reports of its action as an antioxidant (De and Agarwal, 1989; De and Ghosh, 1992). On the other hand, there is also evidence that it may act as a genotoxic agent (Agarwal and Bhide, 1986; Jang and Kim, 1988). In view of such conflicting evidence, I have examined the prooxidant role of capsaicin and have shown that in agreement with the above polyphenolics, it is also capable of causing strand breakage in DNA in the presence of Cu (II) which is known to be a normal component of chromatin. Further, I have also compared both the antioxidant and the prooxidant action of capsaicin with dihydrocapsaicin which is also a constituent of red chili peppers. These studies constitute the second chapter of results.

The result indicate that in the presence of Cu(II) both capsaicin and dihydrocapsaicin act as prooxidants leading to oxidative cleavage. In this respect capuchin is considerably more active in degrading DNA than dihydrocapsaicin. Inhibition of DNA cleavage by Cu(I) specific sequestering agents, neocuproine and bathocuproine, confirms the formation of Cu(I) as an essential intermediate.

Inhibition of DNA breakage by azide, thiourea and benzoate indicated the role of active oxygen species in the DNA cleavage reaction. These compounds were also able to directly produce varying amounts of hydroxyl radicals and hydrogen peroxide. Fluorescence quenching experiments indicated that the compound are capable of binding to DNA and Cu(II). In order to examine the biological activity of the DNA cleavage reaction, experiments were also done to study the viability of bacteriophage lambda on reaction with capsaicin/dihydrocapsaicin-Cu(II). Increasing compound concentrations resulted in a progressive loss of survival of phage. The effect of UV irradiation of host cells on the capsaicin/dihydrocapsaicin-Cu(II) mediated sensitivity of phage indicated that the treatment enhanced the recovery of phage, indicating the involvement of UV-inducible pathway in the repair of DNA. Further, the results of Chapter II support the idea that capsaicin I double edged sword possessing toxic as well as beneficial properties. I have shown that it can act as both as a prooxidant as well as an antioxidant and thus it possess properties similar to other plant derived antioxidants. On the basis of the result obtained, a model for copper binding with capsaicin has been proposed.

Introduction

The food that we eat has a very significant bearing on our physical, mental and spiritual health. According to Ames (1983), the human diet contains a variety of naturally occurring mutagens and carcinogens. The preponderance of certain foods in some countries has been linked to the incidence of certain specific types of cancers in their populations. It is for this reason that dietary mutagens have attracted considerable attention and a number of studies have been undertaken to elucidate the link between dietary practices and cancer. The differences in cancer rates of various population groups are generally ascribed to environmental and life style factors such as smoking, dietary carcinogens and promoters. These differences may be also, to a large extent due to insufficient amounts of anti-carcinogens and other preventive factors in the diet (Maugh, 1979). Studies have suggested that a greater intake of fiber rich cereals, fruits and vegetables and a lower consumption of fat rich products and alcohol is advisable (Doll and Peto, 1981; Peto and Schneiderman, 1981).

In the recent years, a large number of dietary components and constituents have been evaluated in microbial and animal test systems. However, there is still a lack of definitive evidence about their carcinogenecity and mechanisms of action. A large number of chemical carcinogens and mutagens are known to form covalent adducts with DNA and there is a large body of evidence implicating DNA as a critical target in chemically induced cancer (Miller, 1978; O'Connor, 1981). To understand carcinogenesis at the molecular level it is essential to determine the conformational changes in the target macromolecules and relate the

findings to probable aberrations in the functioning of modified macromolecules. In the recent years, there is a growing interest in oxygen radicals and lipid peroxidation as a source of damage to DNA and subsequent promotion of cancer (Harman, 1981; Gensler and Bernstein, 1981; Totter, 1980; Tappel, 1980). In addition, emphasis has also been given to the fact that DNA damage may be caused by endogenous metabolites produced during the bodies normal metabolic processes (Burcham and Marnett, 1994). The mammalian systems have evolved a plethora of defense mechanism against mutagens and carcinogens, the most important of which are against oxygen radicals and lipid peroxidation.

There is growing recognition that cancer induction by occupational and industrial factors accounts for a relatively small percentage of all human cancers. It has been suggested by Doll and Peto (1981) that about 35% of all cancer deaths in the United States may be linked to the diet. According to them, there are five possible ways by which diet may effect the incidence of cancer:

i) ingestion of powerful direct acting carcinogens or their precursors

ii) affecting the formation of carcinogens in the body

iii) affecting transport, activation or deactivation of carcinogens

iv) affecting "promotion" of cells that are already initiated ; and

v) overnutrition

Normal individual consumption of potentially mutagenic substances per day from foods and beverages is estimated to be between 1-2 gm. In addition, the

endogenous conditions favour the formation of still more mutagens *in vivo* in humans (Ohshima and Bartsch, 1981).

MUTAGENS AND CARCINOGENS IN DIETARY PLANT MATERIALS

It is obvious that food is a very complex substance to which humans are exposed. Most people perceive food substances of natural origin as free of risk. Such acceptance is largely based on faith because our objective knowledge on this topic is relatively poor. A large number of chemicals are synthesized by plants, presumably as a defense against a variety of invasive organisms such as bacteria, fungi and insects (Kapadia, 1982; Clarke, 1982; Pamukcu *et al.*, 1980; Stich *et al.*, 1981a). The number of these toxic chemicals is extremely large and new plant chemicals are being continuously discovered (Jadhav *et al.*, 1981; Griesebach and Ebel, 1978). It has been known for many years that plants show experimental carcinogenic activity for several species and various tissues. Wide use of short term tests for detecting mutagens (Ames, 1979; Stich and San, 1981a) and a number of animal cancer tests on plant substances have contributed to the identification of many natural mutagens and carcinogens in the human diet (Kapadia, 1982). Food mutagens are major xenobiotic, genotoxic substances. They include, as typical examples, aflatoxin B1 from *Aspergillus flavus*, nitrosamines in fermented foods, polycyclic aromatic hydrocarbons in heated foods and heterocyclic amines in heated meat and fish (Sugimura, 1982 and 1986; Ames, 1983). Most of these are metabolically oxidized by cytochrome P_{450} and

then esterified to ultimately reactive forms to produce DNA adducts through electrophilic and nucleophilic reactions (Miller and Miller, 1977). Some examples of most frequently ingested compounds are discussed below.

Safrole and Estragole are related compounds that occur in certain species and essential oils and are weak hepatocarcinogens (Fenaroli, 1971; Guenther and Althausen, 1949). Studies have implicated 1′-hydroxysafrole and 1′-hydroxyestragole as proximate carcinogenic metabolites of safrole and estragole, respectively (Drinkwater *et al.*, 1976; Borchert *et al.*, 1973). Eugenol and anethole are structurally related to safrole and estrogole and are widely used as flavouring agents or as food additives. Black pepper contains small amounts of safrole and large amounts of a closely related compound piperine (Concon *et al.*, 1979). Extracts of black pepper caused tumours in mice at a number of sites at a dose equivalent to 4 mg of dried pepper per day given for 3 months.

Ivie *et al.* (1981) have reported that linear furocoumarins (psoralens), which are widespread in plants of the Umbelliferae family, are potent light activated carcinogens and mutagens. Three of the most common furocoumarins are psoralen, xanthotoxin and bergapten. In addition to Umbelliferae, psoralen also occurs in plants from several other families (Ivie, 1978). Psoralens are potent photosensitizers and highly mutagenic in the presence of activating long wavelength UV light. They readily intercalate into duplex DNA where they form

light induced mono- or di- adducts with pyrimidine bases. Psoralen, in the presence of light, is also effective in producing oxygen radicals (Ya *et al.*, 1982).

Pyrrolizidine alkaloids are naturally occurring carcinogens and have been found in some 50 species of the families Compositae, Boraginaceae and Leguminosae (Schoental, 1982), which are used as foods or herbal remedies. Several of these alkaloids are hepatotoxic and certain hepatotoxic pyrrolizidine alkaloids are also carcinogenic (Hirono *et al.*, 1977; Schoental, 1976). However, a number of these alkaloids have been reported to be mutagenic (Clark, 1960) in *Drosophila* and *Aspergillus* system (Alderson and Clark, 1966). Mori *et al.* (1985) have used a hepatocyte primary culture DNA repair test to screen seventeen pyrrolizidine alkaloids for their DNA damaging property. This test is highly responsive to pyrrolizidine alkaloids (Williams *et al.*, 1980). Among the results obtained by these authors is the indication of a species difference in liver bioactivation of these alkaloids. This implies that there may be species difference in the carcinogenic potential of pyrrolizidine alkaloids.

Edible mushrooms contain various hydrazine derivatives in relatively large amounts. Most hydrazines that have been tested have been found to be carcinogenic and mutagenic. The most common commercial mushroom *Agaricus bisporus* contains about 300 mg of agaritine, the glutamyl derivative of the mutagen 4-hydroxymethyl-phenylhydrazine, per 100 gm of mushrooms as well as smaller amount of the closely related carcinogen N-acetyl-4-

hydroxymethylphenylhydrazine (Toth *et al.*, 1982). Some agaritine is metabolized by the mushroom to a diazonium derivative, which is a potent carcinogen and is also present in the mushroom in smaller amounts. Many hydrazine carcinogens may act by producing oxygen radicals (Hochstein and Jane, 1981).

A number of 1,2-dicarbonyl compounds, e.g. maltole, kojic acid, ethyl maltole, diacetyl and glyoxyl have been found to be mutagenic in the *Salmonella*/microsome assay. Several compounds in this class are of toxicological interest because they occur in various foods. For example, maltole is a product of carbohydrate dehydration and is present in soyabean, coffee and baked cereals such as bread. Kojic acid is a metabolite of many micro-organisms including several fungi used in food production, while diacetyl is an aromatic component of butter, beer, coffee, etc. (Fishbein, 1983).

A number of furans, such as 2-methylfuran, 2,5-dimethylfuran, furfural, 5-methylfurfural and 2-furylmethylketone are found in numerous food products including meat, milk products, various nuts, tea and coffee (Maga, 1979). Stich *et al.* (1981b) have reported that these furans induced relatively high frequencies of chromatid breaks and chromatid exchanges when they were exposed to cultured Chinese hamster ovary (CHO) cells in the absence of a liver microsomal preparation. The clastogenic doses of many of the furans were relatively high (100-3900 ppm), whereas the concentration in food products was relatively low. However, Stich *et al.* (1981b) cautioned that the furans are not the only genotoxic

chemicals in the complex mixture of heated, roasted or boiled food products. Even if the furans do not pose a serious health hazard by themselves due to their small amounts in most food items, they do contribute significantly to the total genotoxicity of many consumable foods and beverages.

In addition to pyrrolizidine alkaloids, certain glycoalkaloids found in potato such as solanine and chaconine have been reported to be highly toxic as they are strong inhibitors of choline esterase (Jadhav *et al.*, 1981). Pyrrolizidine alkaloids and other glycoalkaloids can reach levels that can be lethal to humans in potatoes that are diseased or exposed to light (Katsui *et al.*, 1982).

Cyclopropenoid fatty acids, present in cotton seed and other oils have been reported to be carcinogenic and mitogenic having various toxic effects in farm animals. Among these, sterulic acid and malvalic acid are wide spread in the human diet. They are also potentiators of carcinogenecity of aflatoxins (Hendricks *et al.*, 1980). Human exposure to these fatty acids results from the consumption of products of animals fed on cotton seed. Another major toxin in cotton seed is gossypol which accounts for about 1% of its dry weight. Gossypol causes male sterility through formation of abnormal sperm and is carcinogenic as well (Xue, 1980). It is a potent initiator and also promoter of carcinogenesis in mouse skin (Haroz and Thomassan, 1980). Gossypol has been tested in China as a possible male contraceptive as it is inexpensive and causes sterility during use. Its mode of action as a spermicide is presumably through the production of oxygen radicals.

A number of quinones and their phenolic precursors are found in the human diet and have been shown to be mutagens (Stich *et al.*, 1981b; Brown, 1980; Levin *et al.*, 1982). Quinones are quite toxic as they can act as electrophiles or accept a single electron to yield the semi-quinone radicals which can react directly with DNA or generate superoxide radicals (Morimoto *et al.*, 1983; Kappus and Sies, 1981). Many dietary phenols can autoxidise to quinones generating hydrogen peroxide at the same time. The amounts of these phenols in human diet are appreciable. Catechol, which is mainly derived from metabolism of plant substances, is a potent promoter of carcinogenesis and an inducer of DNA damage (Carmella *et al.*, 1982).

In addition, there are many other dietary compounds that have been shown to be mutagenic and carcinogenic in various test systems. Allylisothiocyanate, a major flavour ingredient of mustard oil, is one of the main toxins of mustard seeds and has been shown to be a carcinogen in rats (Dunnick *et al.*, 1982). Phorbol esters, present in plants of Euphorbiaceae family, are potent promoters of carcinogenesis and cause nasopharyngeal and oesophageal cancers (Hecker, 1981). A variety of carcinogens and mutagens are present in mold contaminated food grains, nuts and fruits. Some of these, such as various aflatoxins, are amongst the most potent carcinogens and mutagens known (Hirono, 1981; Tazima, 1982). Nitrosoamines and other nitroso compounds formed from nitrate and nitrites in food have been directly related to the incidence of stomach and oesophageal cancer. Nitrates are present in large amounts in spinach, radish, lettuce and beans

(Magee, 1982). Although alcohol is not a constituent of a normal human diet, in view of its wide spread use, it would be relevant to mention its toxic role. Alcohol has long been associated with the cancer of mouth, pharynx and liver (Tuyns *et al.*, 1982). Alcohol metabolism generates acetaldehyde, which is a mutagen and possibly a carcinogen (Stich and Rosin, 1983; Campbell and Fantel, 1983). It also generates radicals that produce lipid hydroperoxides and other mutagens and carcinogens (Winston and Cederbaum, 1982; Videla *et al.*, 1982).

ADDITIVES IN FOOD AND COSMETICS

Sodium nitrite is extensively used as a preservative in meat, fish and cheese. A possible formation of nitrosoamines from amines, present in or derived from the diet, occurs by reaction with nitrous acid at acidic pH. In humans, gastric juice attains a pH of nearly 1.0. Such high concentration of hydrogen ions gives rise to the nitrosyl cation NO^+, which is a highly reactive nitrosylating agent. Nitrous acid itself is a known mutagen for various bacterial and fungal cells. Its mutagenecity is presumably related to the deamination of adenine and cytosine (Fishbein *et al.*, 1970). Sodium bisulphite is used as a bacteria inhibitor in a variety of beverages and as a preservative in canned fruits and vegetables. The bisulphite anion reacts, rather specifically, with uracil and cytosine, within single stranded regions of DNA and RNA. It is also mutagenic to bacteria and bacteriophages (Singer, 1983). EDTA and its alkali salts are widely used as sequestrants in various foods. They are useful as antioxidants due to their property

of forming poorly dissociable chelate complexes with trace quantity of metal ions such as copper and iron in fats and oils. EDTA has been shown to induce chromosome aberration and breakage in various plant species.

Sodium chloride in the form of common salt is one of the commonest food additives around the world. However, research has shown that areas where people ingest more sodium chloride show an elevated incidence of gastric cancer (Hirayama, 1968). Such salt intake causes mucous membrane damage to stomach as evidenced by the existence of malondialdehyde in extracts of gastric mucous membranes after sodium chloride administration (Takahashi *et al.*, 1991). It is, therefore, likely that sodium chloride – lipid peroxidation – malondialdehyde – mutation scenario plays a major role in carcinogenesis of the stomach (Sugimura *et al.*, 1996).

Saccharin was synthesized in the last century and since then it has been widely used as an artificial sweetener. Reports on the mutagenecity and carcinogenecity of saccharin are conflicting and there is some suggestion that these activities are thought to be due to impurities present in saccharin preparations (Kramers, 1975). The possibility of an *in vivo* conversion of saccharin into a mutagenic metabolite has also been suggested (Batzinger *et al.*, 1977). Another artificial sweetener, which was widely used but is now banned in USA and many other countries, is cyclamate. Cyclamate induces chromosome breakage in cells of several plants and animal species. It is converted *in vivo* into

cyclohexylamine, which is also an inducer of chromosome breaks (Fishbein *et al.*, 1970).

Under normal atmospheric conditions fats and oils slowly become oxidized. Antioxidants such as octyl gallate, prevent this oxidation process. They are also added to other non-fat foods such as cut foods to prevent discolouration (Vander Heijden *et al.*, 1986). Typical products containing octyl gallate include the following: fats, oils and margarine, dried milk, peanut butter, snack foods, chewing gum and soap bases.

Octyl gallate in peanut butter has reportedly caused contact dermatitis in a man whose occupation involved mixing peanut butter with octyl gallate (Van Ketal, 1978). Allergic reactions to other gallic acid esters have also been reported. These include allergic contact dermatitis due to propyl gallate in lip balm (Wilson *et al.*, 1989), lip stick (Cronin, 1980) and other topically applied cosmetics (Heine, 1988). There is a reported case of allergy to octyl gallate causing stomatitis (Pemberton *et al.*, 1993). The eight gallates investigated upto now (methyl, ethyl, propyl gallate etc.) by experimental sensitization are moderate to strong contact sensitizers. Also an increase of side chain length is closely correlated with an increase in sensitizing potential. If the gallates come to be used to a greater extent in topical products because they are more effective than other antioxidants and possibly due to the fact that propyl gallate has excellent light protective properties,

we will certainly encounter more cases of gallate hypersensitivity in the future (Hausen and Beyer, 1992).

OXYGEN FREE RADICALS AND HUMAN DISEASES

Oxygen is an essential element for aerobes as it is the terminal acceptor of electrons during respiration, which is the main source of energy in these organisms. The generation of highly reactive oxygen metabolites is am integral part of the normal cellular metabolism that includes mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism, ovulation and fertilization (Roth, 1997). It has been proposed that known harmful effects of oxygen were due to the formation of free radicals derived from it (Gilbert, 1981). The presence of free radicals can be advantageous for cells. Infact they are being continuously produced in organisms and many of them are necessary to carry out certain biological reactions. For example, recent research has indicated that reactive oxygen species (ROS) can directly affect the cellular signalling apparatus and thereby control of gene expression. It further illustrates the role of ROS as potential inter and intracellular signalling molecules (Palmer and Paulson, 1997). In addition, hydroxyl radicals are thought to act as site specific oxidants in plants, targeted to play a role in loosening the cell wall during cell expansion, fruit ripening and organ abscission (Fry, 1998). However, when there is a free radical over-production or antioxidant defense systems are weakened for any reason,

cellular damage can appear (Valenzeula and Videla, 1989; Halliwell *et al.*, 1992). Cellular DNA may be subjected to oxidative damage resulting from attack by free radicals of exogenous or endogenous origin (Collins *et al.*, 1997). Ionizing radiations, tobacco smoke, pesticides, pollutants or some medications are exogenous sources of free radicals. Intracellular systems also produce oxygen free radicals. The autooxidation of small soluble molecules such as catecholamines, flavins, tetrahydopterins, quinones and thiols in the cellular cytoplasm may produce oxygen free radicals by concomitant O_2 reduction (Fridovich, 1983; Proctor and Reynolds, 1984). Reduced flavins and ascorbic acid upon autooxidation produce superoxide anion. This radical further accepts an electron from a reducing agent, such as thiols to yield peroxide (H_2O_2). There is *in vitro* evidence that H_2O_2 may then react with certain chelates of copper and iron to yield the highly reactive hydroxyl free radical ($OH^{\bullet}$) (Wolff *et al.*, 1986). That the superoxide anion actually appears in metabolism is confirmed by the ubiquitous occurrence of superoxide dismutase. Several cytoplasmic enzymes, for example, xanthine oxidase and aldehyde dehydrogenase generate oxygen free radicals as products of their catalytic cycles. Reactions catalyzed by lipooxygenase and cyclooxygenase in the synthesis pathway of leukotrienes, thromboxanes and prostaglandins involve oxygen free radical production. Cyclooxygenase is also able to metabolize certain xenobiotics to more toxic species which may react with oxygen and yield very reactive oxygen species (Yamammoto, 1991; Riendeau *et al.*, 1989). A main source of superoxide anion ($O_2^{\bullet-}$) is the respiratory burst of phagocytic cells when they are activated. Compounds that stimulate the

biosynthesis of peroxisomes induce over-production of hydrogen peroxide. Peroxisomes have a great capacity to form hydrogen peroxide because they contain a high concentration of oxidases (Brunk and Cadenas, 1988; Del Rio *et al.*, 1992). When the respiratory chain is highly reduced and its activity is dependent on ADP availability, radicals may be formed at sites different from cytochrome oxidase. Data show that autooxidation of ubiquinone and NADH dehydrogenase produces superoxide radicals (Beyer, 1990; Freeman and Grapo, 1982). Cytochromes P_{450} and B_5 of the microsomic electron transport systems generate superoxide radicals during their catalytic cycle. Cytochrome reductases involved in redox reactions of cytochromes P_{450} and B_5 can also produce superoxide radicals and hydrogen peroxide when they undergo autooxidation (Sevanian *et al.*, 1990). The high reactivity of free radicals results in their having a short half-life, as well as a short radius for action. These free radicals react in a way that long chains of propagation are established causing biological effects far from the system which produce the first radical. All the cellular components, lipids, proteins, nucleic acids and carbohydrates may be damaged by reactions with oxygen free radicals, giving rise to metabolic and cellular disturbances.

Oxygen free radicals have been shown to oxidize lipids. Hydroxyl and hydroperoxyl radicals, but neither superoxide nor hydrogen peroxide, are able to attack unsaturated fatty acids of phospholipids and other membrane lipid compounds initiating, in this way, lipid peroxidation. Lipid peroxidation causes severe damage to the membrane structure and, consequently, alters its fluidity and ability to function correctly (Gutteridge and Halliwell, 1990; Niki *et al.*, 1991;

Schaich, 1992). Alcohols, aldehydes, volatile hydrocarbons and hydroperoxides, the final products of peroxidation, inhibit protein synthesis and are also able to alter vascular permeability, inflammatory response and chemotactic activity (Southorn and Powis, 1988; Blake *et al.*, 1987; Del Maestro *et al.*, 1981). In addition, malondialdehyde, a by-product resulting from peroxidation of fatty acids with three or more double bonds and a main indicator of lipid peroxidation has also been found to cause cross-linking and polymerization of membrane components as well as to react with DNA nitrogenated bases (Nielson, 1981; Valenzuela, 1991).

Oxidized proteins increase their hydrophobicity and sensitivity to proteolysis. Free radicals may react with amino acids containing unsaturated or sulfur groups. These reactions give rise to structural disturbances in proteins as well as cross-linking and aggregation phenomena, which are favoured by inter and intra molecular disulfide bond formation (Gebicki and Gebicki, 1993; Stadtman, 1992). Proteins are fragmented by free radicals involving peptide bond hydrolysis following oxidation of proline residues by hydroxyl radical and superoxide anion (Wolff and Dean, 1986). Oxidative radical damage occurs to a large extent in nucleic acids through alterations in both their bases and their deoxyribose sugars. The components of DNA most susceptible to free radical action are the thymine and cytosine bases followed by adenine and guanine and finally the deoxyribose sugar. However, for double stranded DNA, the deoxyribose moiety is modified more frequently than the bases, due to its external location (Davies *et al.*, 1990; Dample, 1990). Carbohydrates are also targets of oxygen free radicals.

Consequently glycosylated proteins are more sensitive to oxygen damage (Freeman and Grapo, 1982; Sies, 1985).

There are many diseases that involve radical reactions in mammalian systems. According to Foga *et al.* (1997), ROS may play an important role in HIV-1 pathogenesis and HIV-1 gp120 induced neurotoxicity. Free radicals also play a major part in inflammation (Randerrath *et al.*, 1992; Kunz *et al.*, 1991), the process, which is the response of the host organism to injury. It involves enhanced vascular permeability with edema formation and leukocyte infiltration into the damaged area. Inflammatory response is advantageous for organisms. However, abnormal overactivation of phagocytes with consequent exacerbation of reactive oxygen metabolite production may damage surrounding tissues and change the viscosity of the extracellular fluid. This is the case in gout and autoimmune diseases such as myasthenia gravis, systemic lupus erythematosus, dermatomyositis etc. (Southorn and Powis, 1988; Halliwell and Gutteridge, 1989). Patients with rheumatoid arthritis suffer from neutrophil accumulation in their joints (Greenwald and Moy, 1980). These neutrophils overproduce the ROS responsible for the depolymerisation of hyaluronic acid, a glycosaminoglycan necessary for maintaining synovial fluid viscosity in joints.

A great number of ocular complaints are associated with oxidative damage. Amongst the components of the eye, the retina is the most sensitive to free radical oxidations. This is due to the fact that macular membranes in the retina have the highest concentrations of polyunsaturated fatty acids of any known

tissue. Furthermore, not only is oxygen turnover in retina very high but mitochondria are abundant in its cells. These factors render the macula highly susceptible to free radical damage, particularly to lipid peroxidation (Gerster, 1991). Retrolental fibroplasia (retinopathy of prematurity) is a complication derived from use of increased oxygen tensions in incubators for premature babies. Hyperoxia inhibits the growth of the retinal blood vessels. The lipid peroxidation and an increased production of thromboxanes A_2 induced by free radicals may be responsible for this condition.

The lung is an organ greatly effected by free radical production. Long periods of exposure to high oxygen pressures damage the lungs of different animal species, causing many diseases (pulmonary emphysema, bronchopulmonary dysplasia, adult respiratory distress syndrome) and even death (Webster and Nunn, 1988; Jackson, 1985). Oxygen free radicals and other toxic products formed by the lung cells themselves and by activated neutrophils that accumulate in the lungs when pure oxygen is breathed, may possibly contribute to the hyperoxidant damage. Inflammation in lungs is characterised by an influx of polymorphonuclear leukocytes (PMN) that release a variety of reactive oxygen species. These ROS have been implicated in epithelial cell DNA damage (Knapen *et al.*, 1999). Tobacco has been suggested as being a contributory factor in the appearance of lung pathologies. Smoking impairs the ability of antiprotease to protect lung elastin from neutrophil proteases because free radicals contained in cigarette smoke inactivate this protein. The final result is the destruction of lung connective tissue elastin (Pryor and Dooley, 1985). Studies suggest that oxygen

free radicals might also be involved in the development of atherosclerotic plaques and ischemia-repufusion injuries (Lehr *et al.*, 1992; Gulati *et al.*, 1992).

Number of theories have been proposed to explain the nature of aging and one such is the free radical theory (Sohal, 1993; Seppi *et al.*, 1991; Ji *et al.*, 1990). According to the free radical theory of aging, these very reactive species, produced continuously during normal metabolism, eventually accumulate, damaging DNA and other macromolecules. This is due to progressive defects in the defense systems against reactions that generate free radicals. The result is the appearance of degenerative lesions and cellular death. Then the organism ages and finally dies.

It has been suggested that certain promoters of carcinogenesis act by generation of oxygen radicals, this being a common property of these substances. It is also been suggested that oxidative macromolecular damage may play a role in the teratologic mechanism of xenobiotics that are bioactivated to a reactive intermediate (Wells *et al.*, 1997). Fat and hydrogen peroxide are among the most potent promoters of carcinogenesis (Welsch and Ayslworth, 1983). Other well-known cancer promoters are lead, calcium, phorbol esters, asbestos and various quinones. Many carcinogens that do not require the action of promoters and are by themselves able to induce carcinogenesis (complete carcinogens), also produce oxygen radicals (Demopoulos *et al.*, 1980). These include nitroso compounds, hydrazines, quinones and polycyclic hydrocarbons. Much of the toxic effect of ionizing radiation damage to DNA is also due to the formation of oxygen radicals

(Totter, 1980). The mechanism of action of promoters involves the expression of recessive genes and an increase in gene copy number through chromosome breaks and creation of hemizygosity (Kinsella, 1982; Varshavsky, 1981). Promoters also cause modification of prostaglandins which are intimately involved in cell division, differentiation and tumour growth (Fischer *et al.*, 1982). Most data on radical damage to biological macromolecules concern with the effects of radiation on nucleic acids because of the possible genetic effects.

ANTICARCINOGENS

One of the theories of etiology of cancer holds that the major cause is due to damage to DNA by oxygen radicals and lipid peroxidation (Ames, 1983; Totter, 1980). The protective defense mechanisms against mutagens and carcinogens include the shedding of surface layer of the skin, cornea and the alimentary canal. The major sources of endogenous oxygen radicals are hydrogen peroxide and superoxide that are generated as side products of metabolism (Pryor, 1976-1982). In addition, oxygen radicals also arise from phagocytosis after viral and bacterial infection or in inflammatory reactions (Tauber, 1982). The exogenous oxygen radicals are contributed by a variety of environmental agents (Pryor, 1976-1982). The enzymes that protect cells from oxidative damage are superoxide dismutase (SOD), catalase, D.T.diaphorase (Lind *et al.*, 1982), glutathione peroxidase and glutathione transferases (Warholm *et al.*, 1981).

A substantial body of data from many epidemiological and laboratory studies support the idea that dietary factors have a profound impact on the prevention of many cancers in humans (Block *et al.*, 1992; Stavric, 1994). Diet can modify the pathophysiological processes of various metabolic disorders and can be an effective preventive strategy for various disease processes most of which have known to involve oxidative damage. Both the nutrient and non-nutrient components of the diet have been recognized for their antioxidant and chemopreventive benefits. The possible chemopreventive mechanisms include carcinogen blocking activities, antioxidant/anti-inflammatory activities and antiproliferative / antiprogressive activities. Carcinogenesis blocking activities encompass inhibition of carcinogen uptake, inhibition of carcinogen formation or activation, deactivation or detoxification of carcinogens, prevention of carcinogen binding to DNA and enhancement of the level of fidelity of DNA repair. Antioxidant/anti-inflammatory activity includes scavenging of reactive electrophiles and oxygen radicals and inhibition of arachidonic acid metabolism. Antiproliferative/ antiprogressive activities comprise modulation of signal transduction, modulation of hormonal and growth factor activity, inhibition of polyamine metabolism, induction of terminal differentiation, restoration of immune responses, enhancement of inter cellular communication, restoration of tumour suppressor function, induction of apoptosis, telomerase inhibition, correction of DNA methylation imbalances, inhibition of angiogenesis, inhibition of basement membrane and activation of antimetastasis genes (Krishnaswamy and

Raghuramulu, 1998; Kelloff *et al.*, 1996). Some small molecules in the human diet act as antioxidative agents and presumably have an anticarcinogenic effect. Some of these compounds are discussed below.

Resveratrol, a phytoalexin found in grapes and other food products has been shown to possess chemopreventive activity. Recent studies have shown that resveratrol acts as antioxidant and antimutagen and mediates anti-inflammatory effects besides inhibiting cyclo-oxygenases and hydroperoxide functions. In addition, it inhibits the development of preneoplastic lesions in carcinogenic treated cells in various cultures (Jang *et al.*, 1997). *Tocopherol* (vitamin E) is an important trap of oxygen radicals in membranes (Pryor, 1976-1982) and has been shown to decrease the carcinogenic effect of quinones, adriamycin and daunomycin which are toxic because of free radical generation (Ames, 1983). Protective effect of tocopherols against radiation induced DNA damage and dimethylhydrazine induced carcinogenesis have also been observed (Beckman *et al.*, 1982). *β-Carotene* is a potent antioxidant present in the diet and is important in protecting lipid membranes against oxidation. Singlet oxygen is a highly reactive form of oxygen which is mutagenic and is mainly generated by pigment mediated transfer of energy of light to oxygen. Carotenoids are free radical traps and are remarkably efficient as quenchers of singlet oxygen (Packer *et al.*, 1981). β-Carotene and similar polyprenes are also the main defense in plants against singlet oxygen generated as a by-product of the interaction of light and chlorophyll (Krinsky and Deneke, 1982). Carotenoids have been shown to be

anticarcinogens in rats and mice and may also have a similar effect in humans (Mathews-Roth, 1982; Peto and Schneiderman, 1981). *Glutathione* is present in food and is one of the major antioxidants and is antimutagenic in cells. Dietary glutathione is an effective anticarcinogen against aflatoxins (Novi, 1981). The cellular concentration of glutathione is influenced by dietary sulfur amino acids (Tateishi *et al.*, 1981). *Selenium,* which is present in the active site of glutathione peroxidase, is another important dietary anticarcinogen. Glutathione peroxidase is essential for destroying lipid hydroperoxides and endogenous hydrogen peroxide and therefore helps to prevent oxygen radical induced lipid peroxidation (Flohe, 1982). Several heavy metal toxins such Cd^{2+} (a known carcinogen) and Hg^{2+} decrease glutathione peroxidase activity by interaction with selenium (Flohe, 1982). Other antioxidants include *uric acid* and *ascorbic acid* The latter has been shown to be anticarcinogenic in rodents treated with UV light and benzo(α)pyrene (Hartman, 1982). Uric acid is present in high concentrations in the blood of humans and is a strong antioxidant (Ames *et al.*, 1981). A low uric acid level has been considered a risk factor in cigarette caused lung cancer, however, too high levels may cause gout.

Flavonoids are widely distributed plant secondary metabolites found in the edible portions of a majority of food plants and beverages. These polyphenolic, low molecular weight compounds possess a wide range of physiological and pharmacological properties which have the potential of being exploited for the therapeutic purpose (Asad *et al.*, 1998). Flavonoids have been reported to possess

antibacterial, anti-inflammatory, antiallergic and vasodilatory activities (Hanasaki *et al.*, 1994; Duarte *et al.*, 1993). In addition, they have also been shown to inhibit lipid peroxidation (Hanasaki *et al.*, 1994; Miyogi *et al.*, 1997), platelet aggregation (Tzeng *et al.*, 1991) and the activity of enzyme systems including cyclo-oxygenases and lipo-oxygenases (Middleton and Kandaswamy, 1993). *Quercetin* in common with other flavonoids is a candidate substance for the development of antiviral agents (Vanden *et al.*, 1986; Van Hoof *et al.*, 1984) and is a promising compound for the inhibition of tumour invasion (Bracke *et al.*, 1987). The mechanism by which quercetin exhibits its antitumour activity is not understood. Since it a frame shift mutagen in *S. typhimurium*.,(Ames, 1972), it has been argued that it might be an intercalating agent (Bjeladanes and Chang, 1977). However, there are no chemical data to support this view. Previous studies have suggested that flavonoids function as scavengers of reactive oxygen species such as singlet oxygen (Takahama, 1984), superoxide anion (Takahama, 1983) and H_2O_2 (Takahama, 1984). From the studies on the effect of metal ions, antioxidants and pH on the mutagenecity of quercetin in *S. typhimurium*, Hatcher and Bryan (1985) concluded that this reaction is antimutagenic.

The polyphenol *Tannic acid* is present in green tea and several plants, including *Embellica phyllanthus*. The latter has been credited with several therapeutic properties in anaemia, jaundice, uremia, cholesterolemia, etc. (Tripathi *et al.*, 1988; Thakur, 1985), some of which may be due to antioxidative properties of tannic acid against DNA damage (Devasagayam *et al.*, 1995) and lipid

peroxidation (Ramnathan and Das, 1992). Flavonoids as well as the related polyphenol tannic acid significantly inhibit single strand breaks in plasmid pBR322 DNA induced by singlet molecular oxygen ($^1O_2$). The protective ability of these compounds were both time and concentration dependent (Devasagayam *et al.*, 1995). Hamamelitannin which contains two galloyl groups and hamamelose has a strong scavenging activity against superoxide anion radicals (Masaki *et al.*, 1993). Studies showed that several flavonoids, tannic acid, gallic acid and fat soluble vitamins inhibited Hela and Raji lymphoma cell growth and are known to possess anticarcinogenic properties (Mukhtar *et al.*, 1988). All tannic acid extracts tested so far are antitumour promoters but their efficacy may vary considerably depending on their origin and length of their polygalloyl chain (Gali *et al.*, 1993).

Capsaicin (trans-8-methyl-N-vanillyl-6-noneamide) is the major pungent principal of hot peppers of the genus *Capsicum* (Park *et al.*, 1998). Apart from its use as a food additive in various spicy cuisines, capsaicin is also utilized for therapeutic purposes to treat various peripheral painful conditions such as rheumatoid arthritis and diabetic neuropathy (Surh and Lee, 1996). Recent studies have revealed various antigenotoxic and anticarcinogenic effects of capsaicin suggesting this compound as another important dietary phytochemical with a potential chemopreventive activity (Surh *et al.*, 1998). Joe and Lokesh (1994) have demonstrated that capsaicin lowers the generation of ROS by activated macrophages. Other workers have demonstrated that capsaicin inhibits cyclophosphamide (CP) induced chromosomal aberrations and DNA strand

breaks. This protective action of capsaicin against CP-induced toxicity may possibly be linked with its already reported desensitization effect against chemical irritant induced damages (De *et al.*, 1995). The antimutagenic and anticarcinogenic properties of capsaicin are believed to be due to its inhibition of xenobiotic metabolizing enzymes (Miller *et al.*, 1993). In several instances it has been reported that capsaicin modulates microsomal cytochrome P_{450} dependent monooxygenase activities, thereby affecting metabolism of carcinogens and other xenobiotics (Miller *et al.*, 1993; Surh *et al.*, 1995; Modly *et al.*, 1986; Teel, 1991; Miller *et al.*, 1993; Zhang *et al.*, 1993). Capsaicin was found to interact with rat hepatic mixed function oxidases as demonstrated by inhibition of ethylmorphine demethylase activity (Miler *et al.*, 1993). The compound also suppresses the activity of rat epidermal aryl hydrocarbon hydroxylase that is responsible for the metabolism of benzo(α)pyrene and other polyaromatic hydrocarbons. Metabolism and subsequent covalent DNA binding of benzo(α)pyrene in human and murine keratinocytes were attenuated by capsaicin. In addition, capsaicin has been shown to display inhibitory effects on metabolism, mutagenecity and/or covalent DNA binding of aflatoxin B_1 and the tobacco specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridal)-1-butanone (NNK) (Zhang *et al.*, 1993; Jang *et al.*, 1989; Teel, 1993). Extracts of hot red peppers also displayed productive effects against aflatoxin B_1 induced bacterial mutagenesis (Kim *et al.*, 1991). Capsaicin, along with its saturated analogue dihydrocapsaicin (DHC) has been shown to inhibit cytochrome $P_{450}2E_1$, an isoform that catalyzes metabolic activation as well as detoxification of many low molecular weight carcinogens

(Guengerich *et al.*, 1991). In agreement with these findings, both compounds inhibited the mutagenecity or tumourogenecity of vinyl carbamate or dimethyl nitrosoamine which are preferentially activated by cytochrome $P_{450}2E_1$. Capsaicin also ameliorated the peroxidative changes in rat hepatic and pulmonary tissues induced by chloroform CCl_4 or dichloromethane (Day and Ghosh, 1992). Capsaicin pretreatment also protects against the free radical induced pulmonary damage in rats exposed to such gaseous chemical irritants as sulfur dioxide and nitrogen dioxide (Day and Ghosh, 1989). Recent studies have revealed that capsaicin induces apoptotic cell death- the most potent natural defense against cancer- in human gastric cancer cells (SNU-1) (Kim *et al.*, 1997).

POSSIBLE ROLE OF ENDOGENOUS SUBSTRATES IN OXIDATIVE DNA DAMAGE

It is well established that aerobic organisms produce oxygen species during the course of normal metabolism. These include superoxide anion, hydrogen peroxide and hydroxyl radical (Fridovich, 1989; Sies, 1991; Weiss, 1989). Oxidative DNA damage by these species has been implicated in a number of human diseases, including cancer. The other diseases where such damage may play an important role are rheumatoid arthritis (Gridley *et al.*, 1993), Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) (Sanchez-Ramos and Ames, 1994; Mecocci *et al.*, 1994; Robberecht *et al.*, 1994). In

addition, oxygen radical toxicity is considered one of the causes of human male infertility (Aitken and Fischer, 1994).

Recent studies have shown that considerable DNA damage may be caused by endogenous metabolites generated during the body's normal metabolic processes (Burcham and Marnett, 1994). For example, it has been shown that malondialdehyde which is the ubiquitous product of lipid peroxidation and eicosonoid metabolism reacts with cellular DNA to form a propanodeoxyguanosine adduct (Chaudhury *et al.*, 1996). Evidence indicates that the adduct exists at significant levels in the hepatic DNA of rats and humans and is an effective premutagenic lesion in *E. coli* (Burcham and Marnett, 1994; Chaudhaury *et al.*, 1994).

In this laboratory studies have been carried out on the mechanism of interaction with DNA of several endogenous metabolites and antioxidants of plant and animal origin. These include flavonoids (Rahman *et al.*, 1989 & 1990; Fazal *et al.*, 1990; Said Ahmad *et al.*, 1992 & 1994), tannic acid (Bhat and Hadi, 1992, 1994a & 1994b; Khan and Hadi, 1998), uric acid (Shamsi and Hadi, 1995), bilirubin (Asad *et al.*, 1999) and L-DOPA (Husain and Hadi, 1995). Of these, uric acid and bilirubin are present in the human extracellular fluid and are considered to have an antioxidant function. However, Stowe and Prutz (1987) have suggested that many biological antioxidants are themselves capable of causing oxidative DNA damage.

L-DOPA (L-3,4-dihydroxyphenylalanine) is an important metabolite in various metabolic reactions. It is formed by the decarboxylation of tyrosine and in turn undergoes decarboxylation to form the neurotransmitter dopamine. Dopamine, which accounts for 90% of the total catecholamines serves as the precursor of hormones adrenalin and noradrenalin. The neurological disorder, Parkinson's disease is associated with an under production of dopamine in the human brain (Lehninger *et al.*, 1993). It is for this reason that L-DOPA is an effective drug in the treatment of Parkinson's disease. Studies have shown, however, that dopamine can condense with acetaldehyde, a product of ethanol metabolism, to generate 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroxyquinoline (Salsolinol) (Dostert *et al.*, 1988). Salsolinol is considered to be involved in the etiology of Parkinson's and Huntington's diseases and has been detected in the cerebrospinal fluid of Parkinsonian patients (Moser and Kompf, 1992). Thus, dopamine may be considered a precursor of an endogenous neurotoxin. Other workers have shown that L-DOPA, dopamine and 3-O-methyl-DOPA caused extensive base modification in DNA in the presence of H_2O_2 and the traces of copper ions (Spencer *et al.*, 1994). These authors have proposed that copper ion release in the presence of L-DOPA and its metabolites may be an important mechanism of toxicity in ALS and Parkinson's disease. Studies in this laboratory has established that L-DOPA in the presence of Cu(II) alone is capable of causing strand scission in DNA *in vitro* and that this breakage results from the generation of reactive oxygen species. That this reaction is biologically active was

demonstrated by the inactivation of Lambda bacteriophage by L-DOPA-Cu(II) system (Husain and Hadi, 1995).

L-DOPA also serves as precursor of melanin, the UV blocking agent in skin. Melanin is synthesized from DOPA by a spontaneous polymerization of DOPA chrome, an oxidised product of DOPA (Nicolaus, 1986). Melanin has been shown to absorb free radicals and active oxygen species (Sarna *et al.*, 1984; Korytowski *et al.*, 1987) but when it does so, free radicals and active oxygen species are also produced. Melanins are unique amongst the biological molecules in that they continuously emit a free radical signal (Sealy, 1984). The free radicals associated with this signal are called "melanin free signals". When melanins are irradiated with UV or when they absorb superoxide anion radical the melanin free signal is enhanced and depending on the conditions, superoxide anion radical, hydroxyl radical and hydrogen peroxide are generated (Korytowski *et al.*, 1987). Melanin mediated radical production is potentially lethal to cells (Menon *et al.*, 1985).

Uric acid is present in human plasma at a relatively high concentration (upto 0.6 mM) and is capable of scavenging hydroxyl radicals, lipid hydroperoxides, singlet oxygen and oxo-heme reductants. In fact, it has been hypothesized that uric acid may be partially responsible for the relatively long life span of humans (Ames *et al.*, 1981). Studies in our laboratory have shown that uric acid in the presence of Cu (II) and molecular oxygen caused DNA breakage

and that this reaction involve active oxygen species such as the hydroxyl radical. Further, uric acid-Cu (II) system was able to inactivate bacteriophage Lambda thereby showing that it is biologically active (Shamsi *et al.*, 1995).

Bilirubin is the end product of heme catabolism. In its bound form with albumin, bilirubin is considered one of the naturally occurring antioxidants of human extracellular fluids (Halliwell and Gutteridge, 1990). It has been the subject of interest because of its toxicity under the conditions of hyperbilirubinemia. The physiological concentration of bilirubin is 5-17 μM (Bloomer *et al.*, 1971). Under certain diseased conditions such as kernicterus and jaundice, the plasma bilirubin levels may reach considerably higher values. Concentrations greater than 300 μM are associated with the risk of development of neurological disorders due to deposition of bilirubin in brain and its enhanced toxic effects on cellular functioning in this tissue (Meuwissen and Heirwagh, 1982; Schenker *et al.*, 1986). Very recent studies in this laboratory have shown that bilirubin forms a complex with Cu(II) and that this complex generates reactive oxygen species due to the redox cycling of the metal ions. The ROS generated, particularly hydroxyl radicals, cause DNA strand breaks *in vitro* (Asad *et al.*, 1999).

Scope of the work presented

It has been proposed that considerable DNA damage may be caused by endogenous metabolites produced during the body's normal metabolic processes. Such reactions are considered to lead to the formation of oxidised bases, alkylated bases and exocyclic bases. Evidence has indicated that exocyclic base adducts exist at significant levels in hepatic DNA of rats and humans and in *E.coli* these are efficient premutagenic lesions. It is considered that such endogenous DNA damages may contribute to the etiology of genetic diseases and may define the baseline of human cancer. As already mentioned, previous studies in this laboratory have shown that L-DOPA causes oxidative DNA damage and that the reaction is catalysed by the formation of hydroxyl radicals. Uric acid is considered to be a naturally occurring antioxidant and is present in the plasma at a relatively higher concentration. In order to further explore the antioxidant functions of uric acid, I have investigated its effect on L-DOPA- Cu (II) mediated DNA cleavage at concentrations similar to or lower than those found in plasma. These studies constitute the first chapter of the results.

The food that we eat has a significant bearing on our overall health. Previous studies in this laboratory have confirmed that several classes of plant derived polyphenolic antioxidant compounds such as flavonoids (Said Ahmad *et al.*, 1992; Fazal *et al.*, 1990) tannins (Bhat and Hadi, 1994; Khan and Hadi, 1994) and stilbenes are themselves capable of oxidative DNA damage in the presence of transitional metal ions. Capsaicin or trans-8-methyl-N-vanillyl-6-noneamide is the principal pungent phenolic substance present in hot red and chili peppers, which

are an important part of various diets around the world. As described above, there are numerous reports of its action as an antioxidant (De and Agarwal, 1989; De and Ghosh, 1992). On the other hand, there is also evidence that it may act as a genotoxic agent (Agarwal and Bhide, 1986; Jang and Kim, 1988). In view of such conflicting evidence, I have examined the prooxidant role of capsaicin and have shown that in agreement with the above polyphenolics, it is also capable of causing strand breakage in DNA in the presence of Cu (II) which is known to be a normal component of chromatin. Further, I have also compared both the antioxidant and the prooxidant action of capsaicin with dihydrocapsaicin which is also a constituent of red chili peppers. These studies constitute the second chapter of results.

Experimental

MATERIALS

Chemicals used for the present study were obtained from the following sources:

Chemicals	Sources
Agar powder	Hi-Media, India
Agarose	Koch light laboratories, England
*Bam*H1	Bangalore Genei, India
Bathocuproine disulphate	Aldrich Chemical Co., USA
BSA	Sigma Chemical Co., USA
Capsaicin	Sigma Chemical Co., USA
Catalase	Sigma Chemical Co., USA
Deoxyribonucleic acid (Calf Thymus Type I)	Sigma Chemical Co., USA
Deoxyribose	SRL, India
Dihydrocapsaicin	Sigma Chemical Co., USA
Diphenylamine	BDH, India
L-DOPA	Fluka AG, Switzerland
*Eco*RI	Bangalore Genei, India
Ethidium bromide	Sigma Chemical Co., USA
Ethylenediaminetetraacetic acid	BDH, India
Hind III	New England Biolabs, England
Histidine	E Merck, Germany

λ DNA	Isolated and purified in the lab according to the procedure of Sambrook *et al.*, 1989.
Mannitol	E Merck, Germany
Neocuproine hydrochloride	Sigma Chemical Co., USA
Nutrient broth	Hi-Media, India
p-nitroso dimethyl aniline	Aldrich chemical Co., USA
Potassium iodide	E Merck, Germany
Psoralen	Sigma Chemical Co., USA
Riboflavin	Sigma Chemical Co., USA
S_1 nuclease	Sigma Chemical Co., USA
Sodium azide	E Merck, Germany
Sodium benzoate	E Merck, Germany
Supercoiled plasmid pBR322 DNA	Isolated and purified in the lab according to the procedure of Sambrook *et al.*, 1989.
Superoxide dismutase	Sigma Chemical Co., USA
Thiobarbituric acid	E Merck, Germany
Thiourea	E Merck, Germany
Tris (hydroxy methyl) aminomethane HCl	Fluka AG, Switzerland

All other chemicals were commercial products of reagent grade

Media for *E.coli* K-12 strains

Nutrient Broth (13.0 g/L) obtained from Hi-Media (India) had the following components-

Peptone	:	5.0 g/L
NaCl	:	5.0 g/L
Beef extract	:	1.5 g/L
Yeast extract	:	1.5 g/L
pH (approx.)	:	7.4 ± 0.2

Nutrient Agar (Hard Agar) :

Nutrient Broth	:	13.0 g/L
Agar Powder	:	15.0 g/L

Soft Agar/Top Agar :

Nutrient Broth	:	13.0 g/L
Agar Powder	:	7.0 g/L

Bacterial Strain

Strain Designation	Genetic Markers	Source
AB 1157	F^-, thr-1, ara-14, len B6, Δ(gpt-proA62), lac Y1, tsx33, $qsr^{/-}$, supE44, galK2, λ rac^-, hisG4(Oc), rfbD1, Mgl-51, rpsL31, KdgK51, xyl-5, mtl-1, argE3(Oc), thi-1	Barbara J. Bachmann, E.coli genetic stock Center, Department of Biology, 3550 ML, Yale University, New Haven, CT, USA

Strain of phage lambda u ed in this study is tabulated as under:

λ_{Vir} Virulent strain; contains an absolute defective mutation in the immunity region and therefore forms clear plaques.

METHODS :

S_1 nuclease digestion of calf thymus DNA in presence of various compounds and Cu (II)

Reaction mixtures (0.5 ml) contained 10 mM Tris-HCl (pH 7.5), 500 μg calf thymus DNA and varying amounts of L-DOPA, capsaicin or dihydrocapsaicin, cupric chloride or other metal ions. Neocuproine, uric acid and free radical scavengers were included in some experiments. All solutions were sterilized before use. After incubation at room temperature for specified time periods, S_1- nuclease digestion was performed. The assay determines the acid soluble nucleotides released from DNA as a result of S_1-nuclease digestion. The reaction mixture in a total volume of 1.0 ml contained 0.1M acetate buffer (pH 4.5), 1mM zinc sulfate, water and enzyme. The mixture was incubated for 2 hours at 48^0C. The reaction was stopped by adding 0.2ml of bovine serum albumin(10 mg/ml) and 1ml of 14% perchloric acid (ice cold). The tubes were immediately transferred to 0^0C for atleast 1 hour before centrifugation to remove the undigested DNA and precipitated protein. Acid soluble nucleotides were determined in the supernatant using the diphenylamine method of Schneider (1957). To a 1.0 ml aliquot, 2.0 ml diphenyl reagent (freshly prepared by dissolving 1g of recrystallized diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc.H_2SO_4) was added. The tubes were heated in a boiling water bath for 20 minutes. The intensity of blue colour was read at 600 nm.

Treatment of supercoiled plasmid pBR 322 DNA with different compounds in the presence of Cu (II).

Reaction mixtures (30 µl) contained 10 mM Tris-HCl (pH 7.5), 0.50 µg of plasmid pBR322 DNA, and other components as indicated in the legends. Incubations were performed for time intervals specified in the legends. After incubation, 10 µl of a solution containing 40 mM EDTA, 0.05% bromophenol blue tracking dye and 50% (v/v) glycerol was added and the solution was subjected to electrophoresis in submarine 1% agarose gels. The gels were stained with ethidium bromide (0.5µg/ml), viewed and photographed on a transilluminator.

Singlet Oxygen monitoring

Formation of singlet oxygen ($^1O_2$) was determined in aqueous solution by the method of Kralijic and Mohsini (1978); p-nitrosodimethylaniline (pRNO) solution was prepared in 0.01 M phosphate buffer (pH 7.8). Histidine (33.3 µg/ml) was added to the pRNO solution as a selective acceptor of singlet oxygen. Irradiation by fluorescent light of the reaction mixture was performed for varying time periods of incubation. Singlet oxygen formed a transannular peroxide intermediate complex with histidine leading to the bleaching of pRNO which was then measured spectrophotometrically at 440 nm. The generation of the singlet oxygen in the reaction system was further established by adding sodium azide to the reaction mixture (Joshi, 1985).

Spectrophotometric study for the reduction of Cu(II) to Cu(I)

The selective sequestering agents neocuproine and bathocuproine were employed to detect reduction of Cu(II) to Cu(I) spectrophotometrically by recording the spectra between 320-600 nm using Beckman DU-40 Spectrophotometer equipped with a plotter. The reaction mixture (1.5 ml) contained 10 mM Tris-HCl (pH 7.5), 0.05 mM capsaicin or dihydrocapsaicin, 0.05 mM Cu(II) and 0.5 mM neocuproine or bathocuproine. The reaction was started by adding Cu(II) and the spectra were recorded immediately after the addition of Cu(II).

Stoichiometric titration of Cu(I) production

The concentration of Cu(I) produced in the compound-Cu(II) reaction mixture was determined by titration with neocuproine/bathocuproine. Neocuproine/ bathocuproine complexes with Cu(I) to form Cu [(neocuproine/bathocuproine)]$_2^+$ complex which has an absorption peak at 450 nm(neocuproine) or 480 nm(bathocuproine)(Nebesar,1961).Briefly,0.005mMof capsaicin/dihydrocapsaicin in 10mM Tris-HCl (pH 7.5) was mixed with varying concentrations of Cu(II) ($CuCl_2$) and neocuproine/bathocuproine, in a total volume of 1.5 ml. Absorbance was recorded at 450 nm in samples containing neocuproine or at 480 nm in samples containing bathocuproine after a 30 minutes incubation at room temperature.

Spectroscopy

The absorption spectra were recorded by using Beckman DU-40 spectrophotometer. The fluorescence spectra were obtained on a Shimadzu spectrofluorometer equipped with a calculator and a plotter. Capsaicin and dihydrocapsaicin were excited at 288 nm and spectra were recorded between 400-600nm.

Determination of Hydroxyl radicals

Two methods were employed to determine hydroxyl radical production by the compounds-

a) Assay of Thiobarbituric acid reactive material

The method of Quinlan and Gutteridge (1987) was followed. The reaction mixtures 0.5 ml contained 2 mM deoxyribose, 50 μM L-DOPA and indicated concentrations of uric acid, thiourea and mannitol. The buffer was 10 mM Tris-HCl (pH 7.5). The reaction was started by adding $CuCl_2$ in sterilized distilled water. and contained increasing concentrations of the compounds and Cu(II) as indicated in the legends. After incubation at 37^0C for the desired time period, the reaction was stopped by adding 0.5 ml of 2.8% (w/v) TCA. Subsequently 0.5 ml of 1% (w/v) thiobarbituric acid (TBA) in 0.05 M NaOH was added and the reaction mixture

was kept in a boiling water bath for 30 minutes. The malondialdehyde-thiobarbiturate adduct was determined colorimetrically at 532 nm.

b) Aromatic hydroxylation of salicylate

This assay is based on the ability of hydroxyl radical to hydroxylate aromatic rings at an almost diffusion controlled rate and the measurement of hydroxylated products by a simple colorimetric method using salicylate (2-hydroxy benzoate) as a detector molecule (Richmond *et al*, 1981). The reaction mixture (2.0 ml) contained the following reagents at the indicated concentrations, 2.0mM salicylate, 0.1 mM EDTA, 0.1mM Cu(II), 150 mM potassium phosphate buffer (pH 8.0) and indicated concentrations of the compounds. The mixture was incubated at 37^0C for the desired time period. After incubation, the reaction was stopped by adding 80 µl of 11.6 M hydrochloric acid and 0.5 g of sodium chloride followed by 4.0 ml chilled diethyl ether. The contents were mixed by vortexing for 1 minute. Next, 3.0 ml of the upper ether layer was pipetted out and evaporated to dryness in a boiling tube at 40^0C. The tubes were cooled and the residue dissolved in 0.25 ml of cold distilled water to which the following reagents were added in the order stated. (a) 0.125 ml of 10% (w/v) TCA dissolved in 0.5 M HCl (b) 0.25 ml of 10% (w/v) sodium tungstate in water and (c) 0.25 ml of 0.5% (w/v) sodium nitrite (freshly prepared). After standing for 5 minutes, 0.5 ml of 0.5 M potassium hydroxide was added and the absorbance at 510 nm was read exactly after 1 minute.

Assay of Hydrogen Peroxide (H_2O_2) production

The production of hydrogen peroxide was measured by the method of Nakayama *et al.*(1983) with modifications. Titanium sulphate solution was prepared from titanium dioxide (Snell and Snell, 1949) and diluted so that the final concentration was 1% (w/v) $Ti(SO_4)_2$ in 1.25 M H_2SO_4. A 2 ml sample containing different amounts of the compounds was mixed with 2 ml of 50 mM sodium phosphate buffer (pH 7.2) and incubated at 37^0C for 30 minutes. An aliquot of the mixture was added to 2 ml of $Ti(SO_4)_2$ solution. A blank sample was also prepared which did not contain $Ti(SO_4)_2$. Absorbance was measured at 410 nm. In order to confirm that the colour change was due to the generation of hydrogen peroxide, in a separate reaction, 0.4 ml of catalase solution (1mg/ml) was added to the reaction mixture before incubation at 37^0C.

Formation of cross links

Plasmid TK-30 DNA was linearized by digestion with *Hin*dIII, precipitated and then dissolved in 10 mM Tris-HCl (pH 7.5) (Sambrook *et al*, 1989). The reaction mixture containing 0.8 µg linearized plasmid DNA, 10 mM Tris-HCl (pH 7 5) and psoralen (0.05 mM) was exposed for UV light for 15 minutes which produces covalent inter strand cross links in DNA. Uric acid/xanthine, capsaicin/dihydrocapsaicin were also included in the reaction mixtures before incubation. Reactions were terminated by addition of an equal volume of 0.6 M

sodium acetate (pH 5.2) and the DNA was precipitated by the addition of 3 volumes of absolute alcohol. Following centrifugation and removal of supernatant the DNA pellet was dried at 37^0C and then dissolved in 30 µl of strand separation buffer (30% dimethyl sulphoxide, 1 mM EDTA and 0.04% bromophenol blue), heated at 90^0C for 2 minutes and chilled immediately in an ice bath prior to loading. Control undenatured sample was dissolved in 30 µl of 6% sucrose, 0.04% bromophenol blue and loaded directly. Electrophoresis was performed on 1% submerged horizontal agarose gel.

Fragmentation of proteins by capsaicin/dihydrocapsaicin in the presence of Cu(II)

The reaction mixture (1 ml) contained 10mM potassium phosphate buffer (pH 8.0), bovine serum albumin (BSA) and varying concentrations of capsaicin and dihydrocapsaicin. The reactions were started by adding $CuCl_2$ in sterilized distilled water. After specified time intervals the reaction mixtures were stopped by adding 1 mM EDTA and precipitated with TCA. The generation of material soluble in 5% TCA was assessed by estimating free amino groups using trinitrobenzene sulphonic acid (TNBS) (Habeeb, 1975). 0.025 ml of 0.03 M aqueous TNBS was added to 0.1 ml of sample and 1 ml of disodium tetra hydroborate buffer (pH 9.5), vortexed to ensure complete mixing and allowed to stand at room temperature for 30 minutes. The reagent blank consisted of 0.025 ml of 0.03 M TNBS in 1.1ml of borate buffer (pH 9.5). The absorbance was read at 420 nm and the reaction was

calibrated with glycine. The principle of this assay is the formation of highly absorbing (A_{420}) chromophore of picrylsulphonamides formed by condensation of the reagent with free amino groups.

Lipid peroxidation induced by Fenton's reagent : effect of different compounds

Lipid peroxidation was induced by the method of Kamat *et al* (1997) with some modifications. The reaction mixture (0.5 ml) contained 10 mM KH_2-PO_4 buffer (150 mM, pH 7.4), 0.125 mM sodium ascorbate solution (pH 7.4), 2.5 μM Fe-SO_4, 5 μM EDTA and 0.0015 % H_2O_2 (Fenton's reagent). 0.1 or 0.2 mM of uric acid/xanthine or capsaicin/dihydrocapsaicin was also added before starting the reaction Incubation was carried out at 37^0C for the specified time periods. After incubation the reaction mixture was boiled for 15 minutes with 1 ml TBA reagent (0.5% TBA in 10% TCA, 6 mM EDTA, 0.63 M HCl). The tubes were then cooled at room temperature and the precipitation formed was removed by centrifugation at 1000xg for 10 minutes. The absorbance of the sample was read at 500nm against the blank that contained all the reagents.

LDL isolation :

Plasma LDL was isolated by a precipitation method described by Wieland *et al.*(1983). The precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 with 5 N HCl and contained 50,000 IU/L Heparin. Before precipitation

of LDL, plasma samples and precipitating reagents were allowed to equilibrate at room temperature (RT). 1ml of the sample was added to 7 ml of heparin-citrate buffer. After mixing, the suspension was allowed to stand for 10 minutes at RT. The insoluble lipoproteins were then sedimented by centrifugation at 1000xg for 10 minutes. The pellet was resuspended in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.9% NaCl.

Maintenance and growth of bacterial strains

Each strain of *E. coli* was streaked on a nutrient agar plate. A single colony was picked up from the plate and repurified by streaking. The culture was tested on the basis of associated genetic markers; raising it from a single colony on a master plate. Having been satisfied with the test clone, the culture was streaked on nutrient agar slants. It was then allowed to grow overnight at 37^0C and stored at 4^0C. the cultures were transferred onto fresh slants every month.

Preparation of bacteriophage lambda and treatment with L-DOPA, capsaicin/dihydrocapsaicin and Cu(II)

Bacteriophage stocks were prepared on plates by confluent lysis method (Sambrook *et al*, 1989). Bacteria from the exponential culture were harvested and resuspended in 10 mM $MgSO_4$ solution. 0.3 ml of AB1157 host cells were infected with phage lambda at a multiplicity of infection (m.o.i) of 5.0. Adsorption was

allowed for 20 minutes at 37^{0}C and plated with 3.0 ml of molten soft agar on hard agar plates. Plates were then incubated at 37^{0}C till confluent lysis was visible to the naked eye. The top agar containing phage lambda was scrapped in a 10 mM $MgSO_4$ solution, 1% chloroform was then added and mixed by gentle vortexing at 37^{0}C. Phage was obtained in the supernatant by centrifugation of the above lysate at 10,000 rpm for 20 minutes at 4^{0}C. The phage stock thus obtained was titred and stored at 4^{0}C over few drops of chloroform.

For assay of phage inactivation, L-DOPA or capsaicin/dihydrocapsaicin and $CuCl_2$, previously sterilized by filtration, were added to the phage suspension (0.25ml) in Tris-HCl / Mg^{2+} buffer (10 mM each, pH 7.5). The reaction mixture was incubated for varying time intervals at 37^{0}C during which it was vortexed at 5 minute intervals. Neocuproine or oxygen free radical quenchers were added before the addition of $CuCl_2$ in some experiments. After incubation, treated phage was diluted with 10 mM $MgSO_4$ and 0.1 ml of diluted phage was added to a 0.3 ml suspension of *E. coli* host strain. Lambda-*E. coli* complexes were vortexed for 1 minute and then incubated for 20 minutes at 37^{0}C. After incubation 3.0 ml of soft agar (40^{0}C) was added to the treated phage, vortexed and immediately poured on the nutrient agar plates. Plates were incubated at 37^{0}C for 5-6 hours followed by counting of plaque forming units (PFU).

Chapter I

Antioxidant effect of uric acid on L-DOPA-Cu(II) mediated DNA cleavage

Results

Breakage of calf thymus DNA by L-DOPA-Cu(II) and effect of uric acid

It has been shown that L-DOPA in the presence of Cu(II) generates single strand breaks in calf thymus DNA (Husain and Hadi, 1995). The breakage and the effect of uric acid thereof was assayed by recording the proportion of double stranded DNA converted to acid soluble nucleotides by S_1-nuclease. Control experiments (data not shown) established that heat denatured DNA (incubated at 48^0C) underwent 100% hydrolysis following the treatment with S_1 nuclease whereas only 9% of native DNA was hydrolysed. Figure 1 gives the kinetics of such an experiment in the absence and presence of 300 μM uric acid. At the end of 4-hour incubation period, a decrease of about 30% in the amount of acid soluble material produced is observed. In order to further substantiate these results, conversion of supercoiled plasmid DNA to relaxed open circles and linear forms was used as an assay. This is a sensitive test for just one nick per molecule. Figure 2 shows the effect of three different concentrations of uric acid on the conversion of supercoiled pBR 322 DNA to open circular and linear forms. It is evident that the formation of linears is inhibited by 200 μM uric acid and completely inhibited at a 400 μM concentration (lane 4). As xanthine is the structural analogue and the metabolic precursor of uric acid, it was also of interest to observe its effect on single strand breakage of plasmid DNA by the L-DOPA-Cu(II) system As can be seen from figure 2, (lanes 6-8), even at 100 μM concentrations, xanthine is able to prevent the full conversion of molecules to the relaxed form (lane 6). Figure 3

Fig 1. Kinetics of degradation of calf thymus DNA by L-DOPA-Cu(II) in the presence (■) and absence (●) of 0.3 mM uric acid.

The concentration of L-DOPA and Cu (II) was 0.1 mM each. All points represent triplicate samples and mean values are plotted. See "Methods" for details.

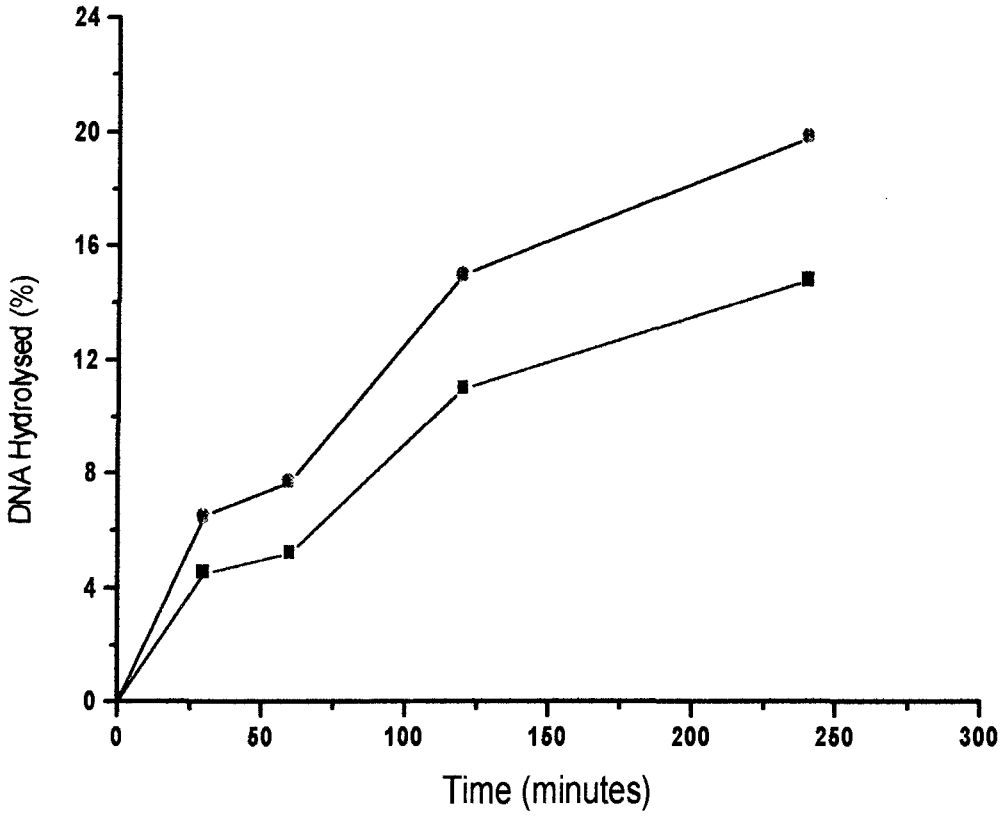

24
20
16
12
8
4
0
DNA Hydrolysed (%)
0
50
100
150
200
250
300
Time (minutes)

Fig 2. Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 DNA showing the effect of various concentrations of uric acid and xanthine on DNA cleavage by L-DOPA-Cu(II).

Lane 1: DNA alone (0.04 μg); Lane 2: L-DOPA+ Cu (II); Lanes 3-5: 0.1, 0.2 and 0.4 mM uric acid and Lanes 6-8: 0.1,0.2 and 0.4 mM xanthine respectively. Concentrations of L-DOPA and Cu(II) were 0.05mM each. Reaction mixtures were incubated at room temperature for 45 minutes.

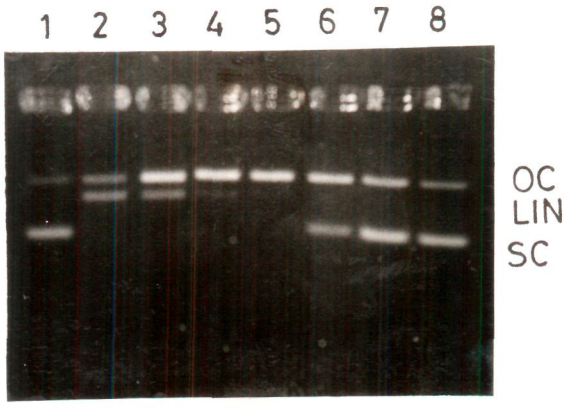
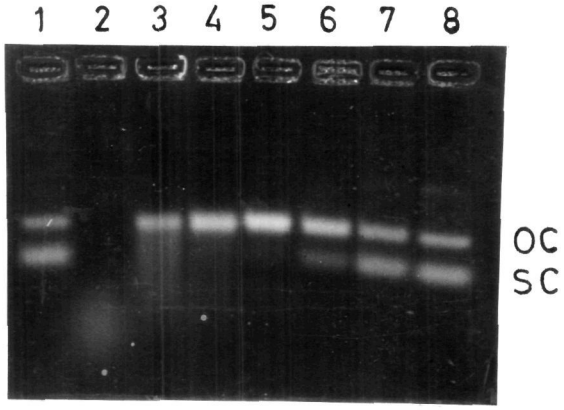

1 2 3 4 5 6 7 8
OC
LIN
SC

Fig 3. Effect of different concentrations of xanthine on the cleavage of supercoiled plasmid DNA by L-DOPA and Cu (II) [0.05 mM each].

Lane 1: DNA alone (0.04 μg); Lane 2: L-DOPA+ Cu (II); Lanes 3-8: 0.01, 0.02 0.05, 01, 0.2 and 0.4 mM xanthine respectively. Reaction mixtures were incubated at room temperature for 1 hour.


1 2 3 4 5 6 7 8
OC
SC

demonstrates the effect of even lower concentrations (10,20 and 50 μM of xanthine on L-DOPA-Cu(II) damage to supercoiled DNA. It is interesting to note that even at xanthine concentration as low as 10 μM there is no formation of linears from relaxed forms.

Previous work by Husain and Hadi (1995) has implicated hydroxyl radicals as a proximal cleaving agent in the oxidative DNA breakage by L-DOPA-Cu(II) system. We have therefore compared the inhibitory effect of uric acid and xanthine with two of the known scavengers of the hydroxyl radical, namely mannitol and thiourea. Results in figure 4 confirm that xanthine exhibits a greater inhibitory effect as compared to uric acid (lanes 3 and 4). However, mannitol and thiourea (lanes 5 and 6) do not show any inhibition of the cleavage at the concentration (200 μM) used for uric acid and xanthine. It may be noted that the concentrations of mannitol and thiourea required for *in vitro* protection against DNA cleavage by L-DOPA-Cu (II) is 50 mM (Husain and Hadi, 1995). This explains the effect of these two scavengers in the above experiments.

Inhibitory effect of uric acid on hydroxyl radical generation

Figure 5 shows the effect of increasing concentration s of L-DOPA on the formation of hydroxylated salicylic acid in the presence and absence of 50 μM uric acid. It is seen that the rate of formation of hydroxyl radicals is substantially

Fig 4. Effect of uric acid, xanthine, mannitol and thiourea on the cleavage of supercoiled plasmid DNA by L-DOPA-Cu(II).

Plasmid DNA was incubated with 50μM L-DOPA-Cu(II) in the presence of 0.2mM each of uric acid, xanthine, mannitol and thiourea. Reaction mixtures were incubated for 45 minutes at room temperature. Lane 1: DNA alone, lane 2: DNA+L-DOPA+ Cu(II). Lanes 3-6: uric acid, xanthine, mannitol and thiourea respectively.

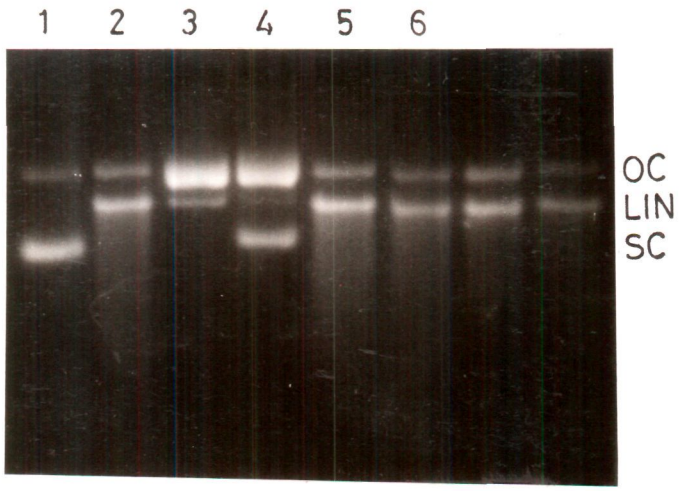
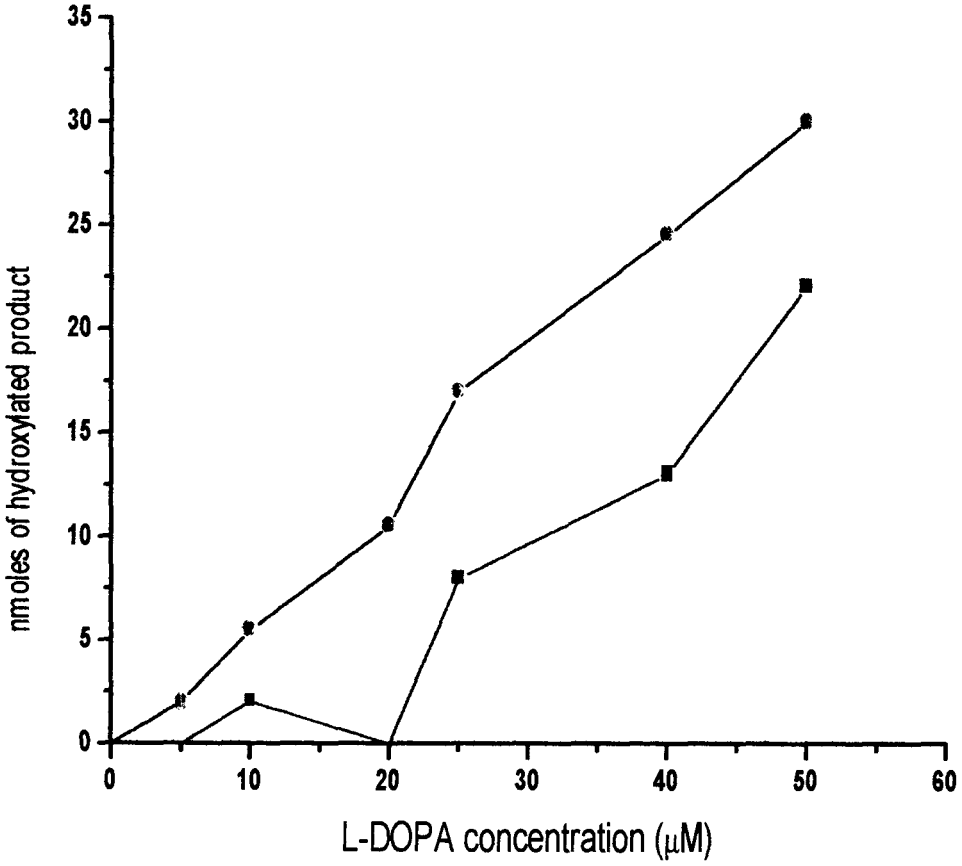

1
2
3
4
5
6
OC
LIN
SC

Fig 5. Effect of uric acid on the formation of hydroxyl radicals by L-DOPA-Cu(II).

Concentration of Cu(II) and uric acid(■) was 0.025 and 0.05mM respectively. Salicylate was used as a reporter molecule. See "Methods" for details.

(■) - Control


35
30
25
20
15
10
5
0
nmoles of hydroxylated product
0
10
20
30
40
50
60
L-DOPA concentration (μM)

reduced in the presence of uric acid and that it remains so at the higher L-DOPA concentration of 50 μM. At this concentration, the inhibition is about 25%. This result indicates that uric acid quenches the hydroxyl radicals generated by L-DOPA-Cu(II) systems.

Generation of hydroxyl radicals by L-DOPA-Cu(II) system: Effect of increasing uric acid concentrations.

We directly tested the effect of uric acid on the generation of hydroxyl radicals by L-DOPA-Cu(II). For this purpose, the assay used involves the reaction of hydroxyl radicals with 2'-deoxyribose molecules. The resulting free radical intermediate decomposes to form an aldehyde which in turn forms an adduct with thiobarbituric acid (TBA). The effect of two concentrations of uric acid (25 and 50 μM) on the kinetics of hydroxyl radical generation was studied and from figure 6 a dose response relationship is observed since the inhibitory effect of 50 μM uric acid (64%) is greater than 25 μM uric acid (45%). The quenching effect of uric acid was also compared with thiourea and mannitol (figure 7). In the presence of uric acid, the rate of formation of hydroxyl radicals was reduced to about 50% at the end of a three hour incubation period. At the same concentration, mannitol did not show any effect whereas thiourea was inhibitory to the extent of 20%.

Fig 6. Comparison of quenching effects of increasing concentrations of uric acid on hydroxyl radical generation by L-DOPA-Cu(II).

Concentrations of L-DOPA and Cu(II) were 0.05 and 0.1mM respectively. Uric acid was present at 50 (■) and 100μM (▲) concentrations.

(●) - Control

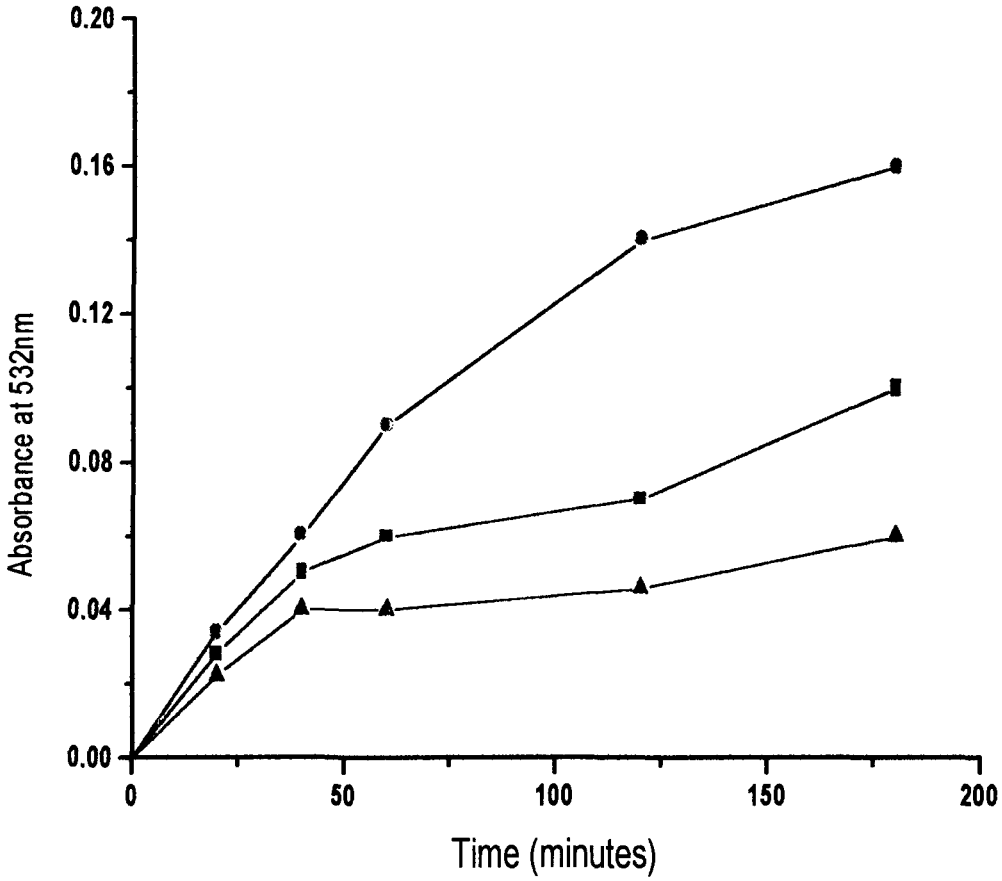
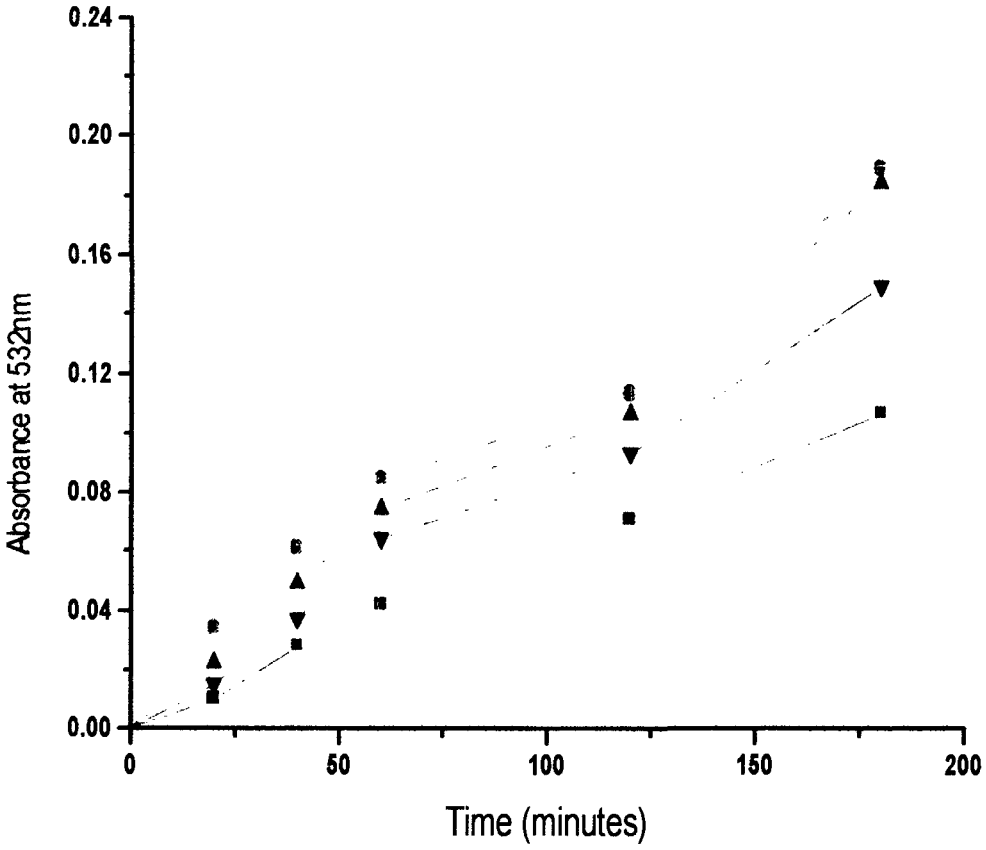

0.20
0.16
0.12
0.08
0.04
0.00
Absorbance at 532nm
0
50
100
150
200
Time (minutes)

Fig 7. Comparison of quenching effect of uric acid(■), mannitol(▲) and thiourea(▼) on hydroxyl radical generation by L-DOPA-Cu(II).

The concentration of L-DOPA and Cu(II) was 50 and 100μM respectively while that of quenchers was 25μM each.

(●) - Control


0.24
0.20
0.16
0.12
0.08
0.04
0.00
Absorbance at 532nm
0
50
100
150
200
Time (minutes)

Fenton's reagent induced low density lipoprotein (LDL) degradation : Inhibition by uric acid and xanthine.

Fig. 8 shows the effect of 0.1 mM uric acid and xanthine on LDL degradation by Fe-EDTA-Ascorbate (Fenton's reagent). The generation of hydroxyl radicals was carried out by the reduction of hydrogen peroxide. As can be seen, there is significant inhibition by both uric acid and its structural analogue xanthine. A comparison of the inhibitory efficiency of uric acid and xanthine reveals that xanthine is a better inhibitor of LDL degradation by Fenton's reagent. As the inhibition by xanthine was nearly twice than that by uric acid.

Induction of inter strand cross links in linearized plasmid DNA by psoralen and effect of uric acid.

Psoralens have been found to interact with many biological targets in the presence of light. The most predictable reaction is a (2+2) cycloaddition between a pyrimidine base and one double bond of the psoralen, either in the furan or in the pyrane moiety (Kagan *et al.*, 1992). When a monoadduct is first formed on the furan side, further irradiation of the remaining coumarin chromaphore may also produce a cycloadduct on the pyrene side, generating a cross-linked DNA.

Fig 8. Effect of uric acid and xanthine on LDL degradation by Fenton's reagent (Fe-EDTA) as a function of time.

The system used for the generation of hydroxyl radical contains H_2O_2 (3.75 X 10^{-3}%), sodium ascorbate (0.125mM) and Fe(II)-EDTA (2.5μM Fe(II)-5μM EDTA). A stock solution of Fe(II)-EDTA was prepared immediately before use by mixing equal volumes of freshly prepared 0.4mM Fe(II) [by dissolution of $(NH_4)Fe(SO_4)_2.6H_2O$] and 0.8mM EDTA. LDL equivalent to 100μg protein in 10mM phosphate buffer (pH 7.4) was incubated with this reagent and 0.2mM each of uric acid (■) and xanthine (▲). Incubation was done at 37^0C for 30, 60, 90, 120 and 150min. Post incubation procedure was as given in "Methods".

(●) - Control

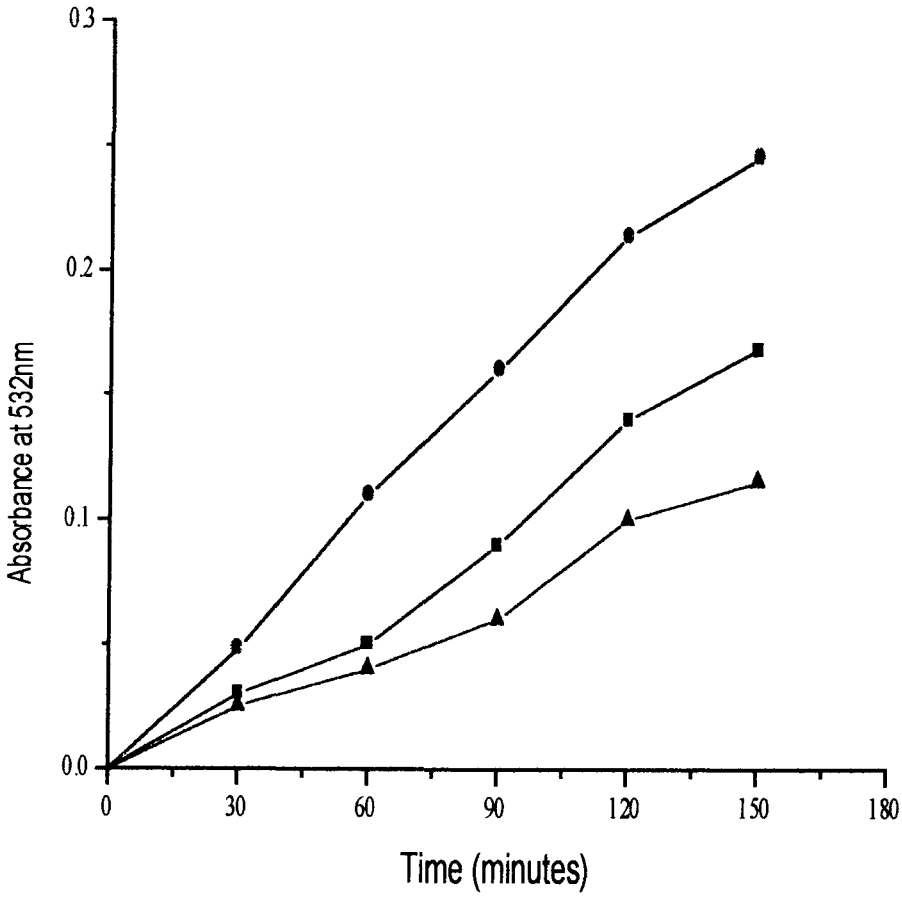
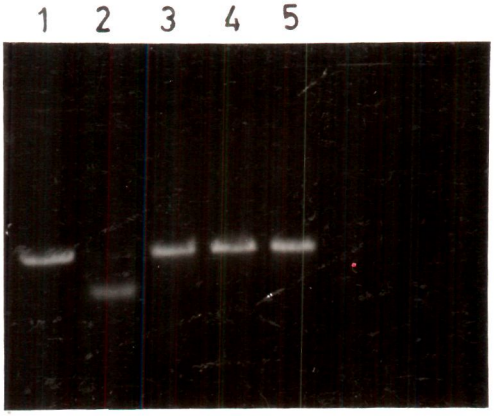

0.3
0.2
0.1
0.0
Absorbance at 532nm
0
30
60
90
120
150
180
Time (minutes)

Figure 9 shows the effect of uric acid and xanthine (0.1 mM each) on the induction of interstrand cross-links by psoralen in TK30 DNA. A fifteen minute incubation of the linearized plasmid with psoralen (0.05 mM) in the presence of UV light leads to the formation of inter strand crosslinks, as revealed by the decreased in the extent of denaturation of the double stranded linear DNA to single stranded form (lane3) As can be seen, the presence of neither uric acid (lane 4) nor xanthine (lane 5) has any effect on the formation of cross links in the linearized, double stranded plasmid.

Fig 9. Effect of uric acid and xanthine on the formation of crosslinks in linearized plasmid DNA pBR322.

Linearized plasmid DNA (0.8μg) was incubated with 0.05mM psoralen for 15 minutes under UV light. Details of the experiment are given in "Methods". Lane 1 : DNA alone; Lane 2 : denatured DNA ; Lane 3 : DNA + psoralen ; Lane 4 : DNA + psoralen + 0.1mM uric acid ; Lane 5 : DNA + psoralen + 0.1mM xanthine.


1
2
3
4
5

Discussion

L-DOPA is an endogenous metabolite and is produced from tyrosine by a copper-containing enzyme, namely tyrosine hydroxylase and presents a possibility of copper chelation. Husain and Hadi (1995) have shown that L-DOPA in the presence of Cu (II) causes DNA breakage through the generation of the hydroxyl radical. It was also shown that L-DOPA is capable of binding to DNA. It is recognized by most workers that hydroxyl radical reactions with DNA are preceded by the association of a complex with DNA, followed by the production of hydroxyl radicals at that particular site (Pryor, 1988). Since L-DOPA binds to DNA and if copper ions are available, a ternary complex of L-DOPA-Cu (II)-DNA may be formed as in the case of several other ROS generating and DNA cleaving systems (Wong *et al.*, 1984). Halliwell and coworkers (1994) have shown that copper ion concentration in human brain tissue damaged in Parkinson's disease is at a level that could produce oxidative DNA damage.

A number of molecules in the human extracellular fluid are considered to have antioxidant function (Halliwell and Gutteridge, 1990). These include ascorbic acid and uric acid. Uric acid is present in human plasma at a relatively high concentration and is capable of scavenging hydroxyl radical, lipid hydroperoxides, singlet oxygen and oxo-heme oxidants (Ames, 1981). In order to examine the antioxidant role of uric acid against endogenous DNA damage, I have studied its effects on L-DOPA-Cu (II) mediated DNA breakage. The results indicate that uric

acid inhibits such DNA breakage and quenches the hydroxyl radical production by the same system at concentrations similar or lower to those found in the plasma.

The normal concentration of uric acid in human plasma is 3-9 mg/dl. or 0.2-0.6mM (Murray et al., 1992). Under certain diseased conditions such as hyperuricemia, gout and arthritis, the uric acid concentration is increased two to four times than normal. In the present studies, the inhibitory concentration of uric acid for L-DOPA-Cu (II) mediated DNA cleavage is 0.2-0.4 mM. The concentration required for direct scavenging of L-DOPA- Cu (II) generated hydroxyl radicals is even lower (25-50 μM). Thus, these studies indicate that at the physiological concentrations found in blood, uric acid is capable of exerting a scavenging effect on L-DOPA-Cu (II) generated DNA damaging oxygen radicals. Further, the studies support the putative antioxidant role of uric acid in higher primates.

Having stated the above, it is to be recognized that it is not yet established whether uric acid is present in the cell nucleus. Although it is hydrophilic in nature, it is conceivable that as a complex with copper, uric acid is capable of traversing the cell or nuclear membrane. Uric acid binds metal ions such as copper and iron (Davies *et al.*, 1988). In addition to chromatin, normal serum contains upto 8 μM loosely bound copper (Burkitt *et al.*; Rowley and Halliwell, 1983). Loosely bound copper is defined by Gutteridge as that copper which is available for binding to the chelating agent 1,10- phenanthroline (Gutteridge, 1984). It is possible that such

loosely bound copper is also available for binding to uric acid. Several of the known antioxidants such as flavonoids in plants and ascorbate in animals are known to generate ROS in the presence of transition metal ions and lead to DNA cleavage (Said Ahmad *et al.*, 1994). Similarly, we have shown that uric acid in the presence of Cu (II) is capable of causing strand scission in DNA and that this reaction is associated with the generation of hydroxyl radicals (Shamsi and Hadi, 1995). However, the concentration of uric acid required for any significant DNA cleavage is 1mM. Irrespective of the physiological significance our results, it is suggested that whereas potential endogenous damage may occur, the animal system also provides for preventive mechanisms against such damage.

Chapter II

Studies on the antioxidant and prooxidant action of capsaicin and dihydrocapsaicin

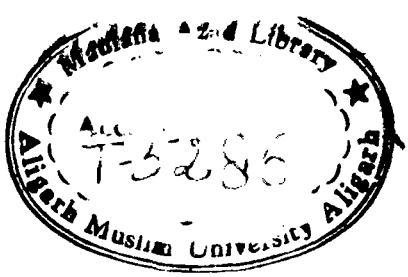
Results

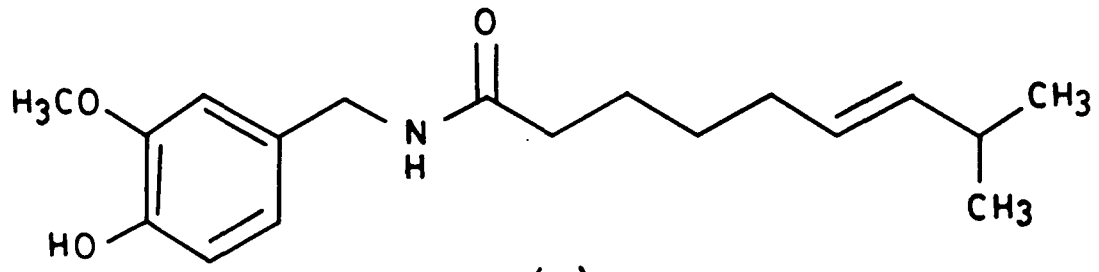
Breakage of calf thymus DNA and cleavage of plasmid DNA by capsaicin and dihydrocapsaicin

Figure 11 gives the rate of S_1-nuclease hydrolysis of calf thymus DNA following damage induced by capsaicin and its saturated structural analog dihydrocapsaicin in presence of Cu(II). The reaction was assayed by recording the proportion of double stranded DNA converted to acid soluble nucleotides by S_1-nuclease. Control experiments (data not shown) established that heat denatured DNA (incubated at 48^0C) underwent 100% hydrolysis following the treatment with S_1-nuclease whereas only 9% of native form was hydrolysed. There is a dose dependent as well as time dependent increase of DNA degradation upon treatment with increasing concentrations of the compounds tested at a fixed concentration of Cu(II). In both cases the rate of DNA degradation by capsaicin is considerably greater than with dihydrocapsaicin.

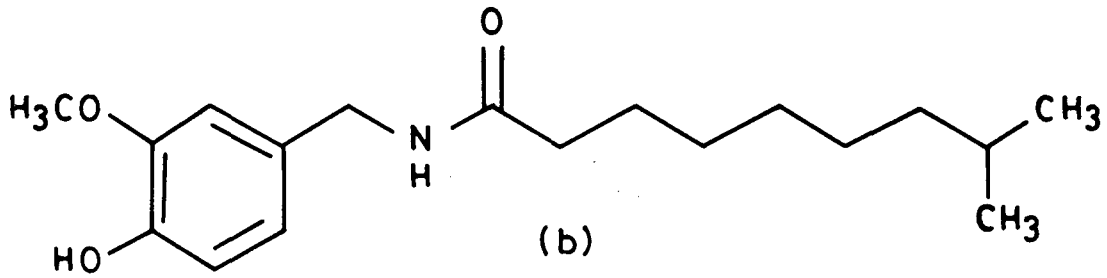
In order to further substantiate these results, conversion of supercoiled plasmid DNA to relaxed open circles and linear forms was used as an assay. This is a sensitive test for just one nick per molecule. Figure12 shows the ethidium bromide stained banding pattern of pBR322 DNA treated with increasing concentrations of capsaicin (lanes 2, 3 and 4 respectively) and dihydrocapsaicin (lanes 5, 6 and 7 respectively) in the presence of 0.5mM Cu(II). As can be seen, at lower concentrations both capsaicin and dihydrocapsaicin show similar banding pattern

Fig 10. Structural formulae of Capsaicin (a) and Dihydrocapsaicin (b).





(a)



(b)

Fig 11. Degradation of calf thymus DNA as a function of increasing capsaicin / dihydrocapsaicin concentration in presence of Cu(II).

DNA was incubated with increasing concentrations of capsaicin [0.1(✖), 0.5(▲) and 1mM(●)] and dihydrocapsaicin [0.1(✚), 0.5(▼) and 1mM(■)] in the presence of 0.5mM Cu(II) at 37^0C for different time intervals. S_1-nuclease digestion and determination of acid soluble material was done as described in "Methods". All points represent triplicate samples and mean values are plotted.

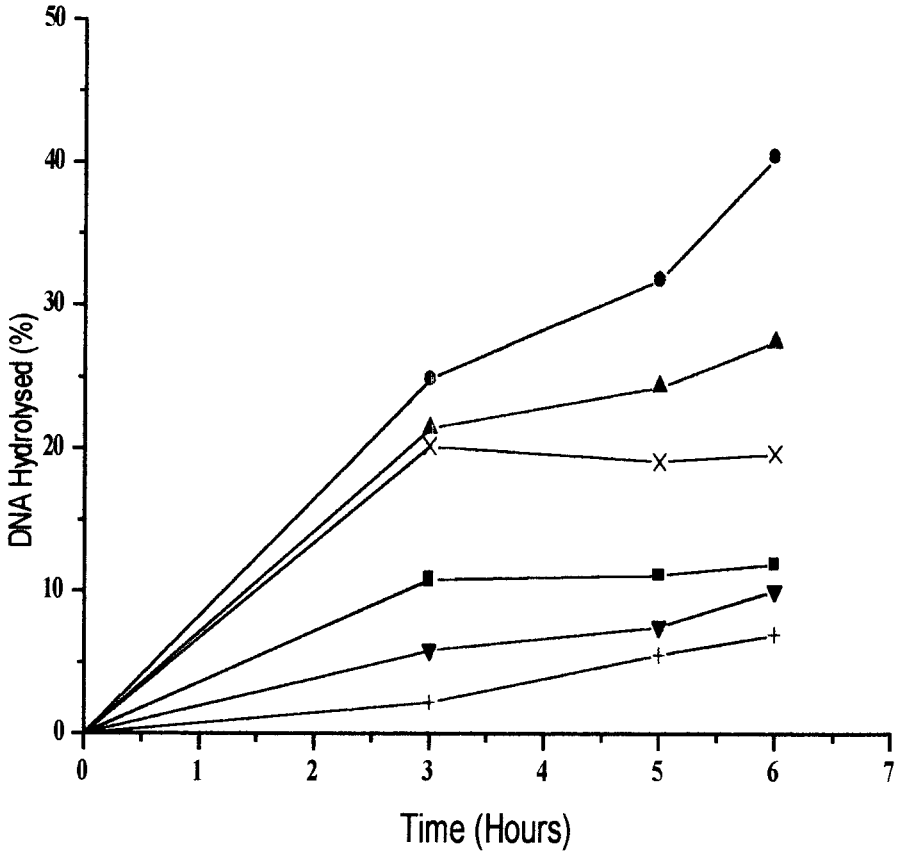
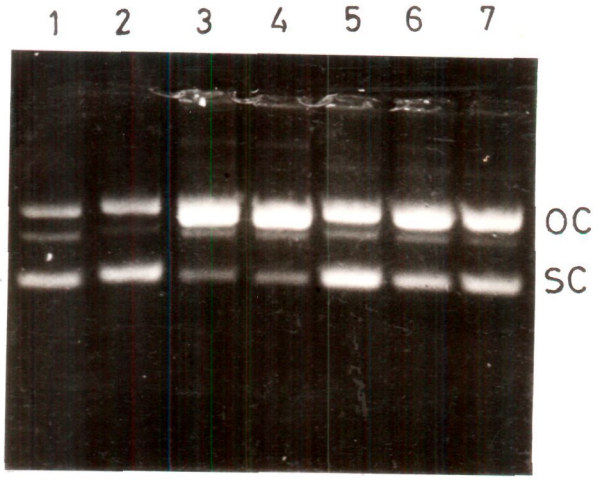

DNA Hydrolysed (%)
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Time (Hours)

Fig 12. Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 DNA showing the effect of increasing concentrations of capsaicin and dihydrocapsaicin in the presence of Cu(II).

Reaction mixtures were incubated at 37^0 C for 5 hrs.

Lane 1: Native DNA; Lane 2: DNA+ Capsaicin (0.1 mM); Lane 3: DNA+ Capsaicin (0.5 mM); Lane 4: DNA+ Capsaicin (1 mM); Lane 5: DNA+ DHC (0.1 mM); Lane 6: DNA + DHC (0.5mM); Lane 7: DNA + DHC (1 mM). 0.5mM Cu(II) was present in all except the first lane.


1
2
3
4
5
6
7
OC
SC

and very little of the supercoiled DNA is converted to open circular form (lanes 2 and 5). At the concentration of 0.5mM (lanes 3 and 6) the intensity of open circular form is greater in case of capsaicin (lane3) than that of dihydrocapsaicin (lane 6). This effect is further enhanced in case of 1mM concentration where intensity of the band corresponding to open circular form is significantly greater in case of capsaicin (lane 4) than dihydrocapsaicin (lane 7). This result corroborates the result of figure 11 and confirms the greater efficiency of capsaicin for DNA cleavage.

Changes in the absorption spectrum by visible light

Both capsaicin and dihydrocapsaicin exhibit a UV absorption spectrum below 320 nm with a major peak absorbing at 277 nm (figure13). Also, dihydrocapsaicin undergoes a photosensitization reaction on exposure to visible light, possibly autooxidation and the absorption spectrum undergoes a quenching effect leading to a decrease in the intensity of the band at 277 nm (figure 14). Capsaicin on the other hand does not show any such effect in visible light.

Reduction of Cu(II) to Cu(I)

Bathocuproine and neocuproine are selective Cu(I) sequestering agents. These compounds were employed to quantitatively detect the reduction of Cu(II) to Cu(I) by capsaicin and dihydrocapsaicin. The Cu(I) chelates of bathocuproine

Fig 13. UV absorption spectrum of capsaicin(_) and dihydrocapsaicin(_ _).

Spectra of 0.1mM compounds in 10mM Tris-HCl (pH 7.5) were recorded.

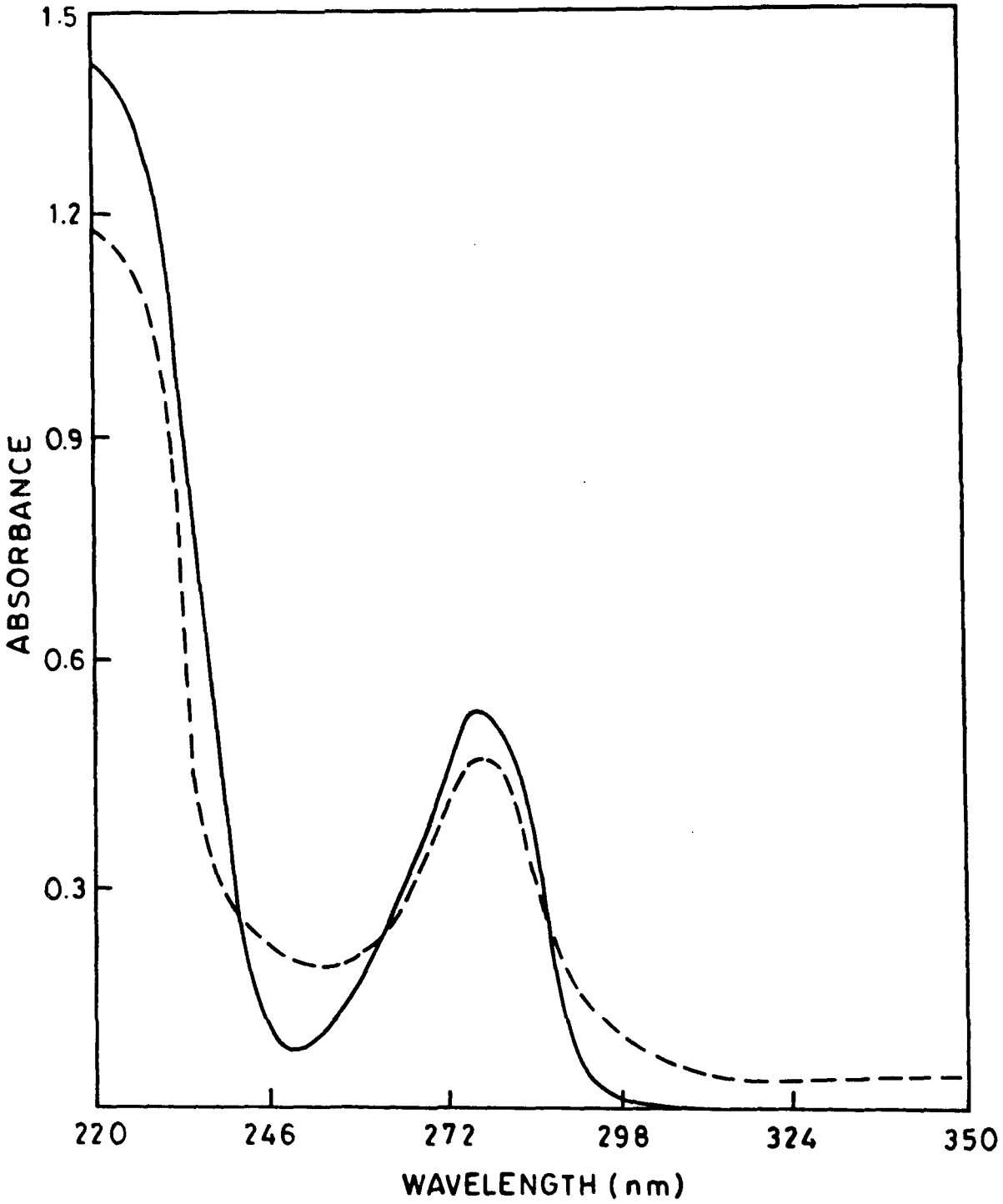
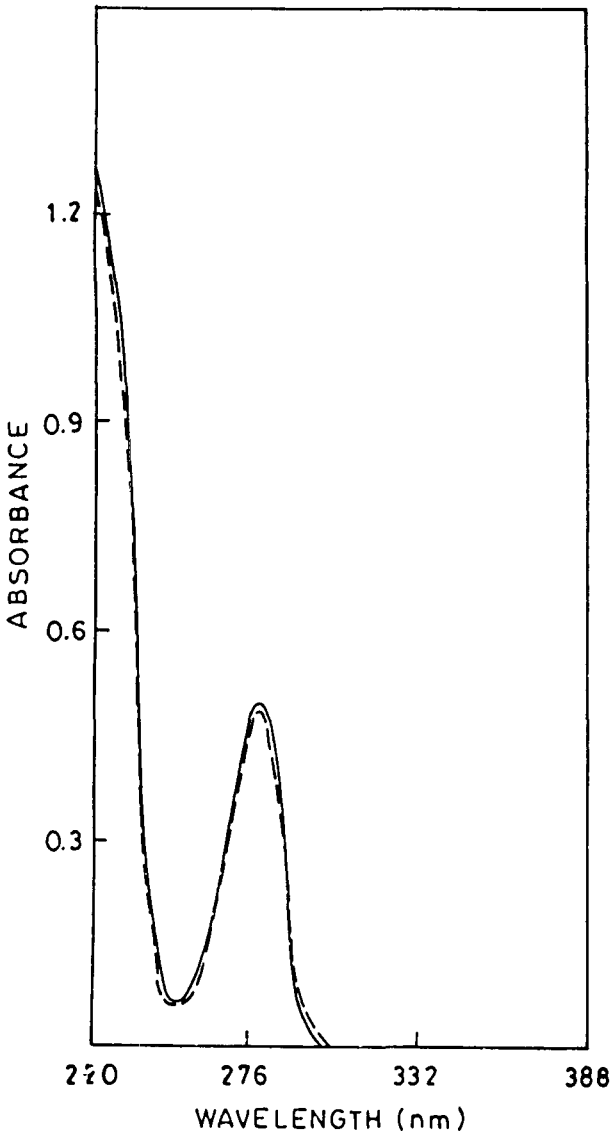

1.5
1.2
0.9
0.6
0.3
ABSORBANCE
220
246
272
298
324
350
WAVELENGTH (nm)

Fig 14. Time course of changes in absorption spectrum of capsaicin (a) and dihydrocapsaicin (b).

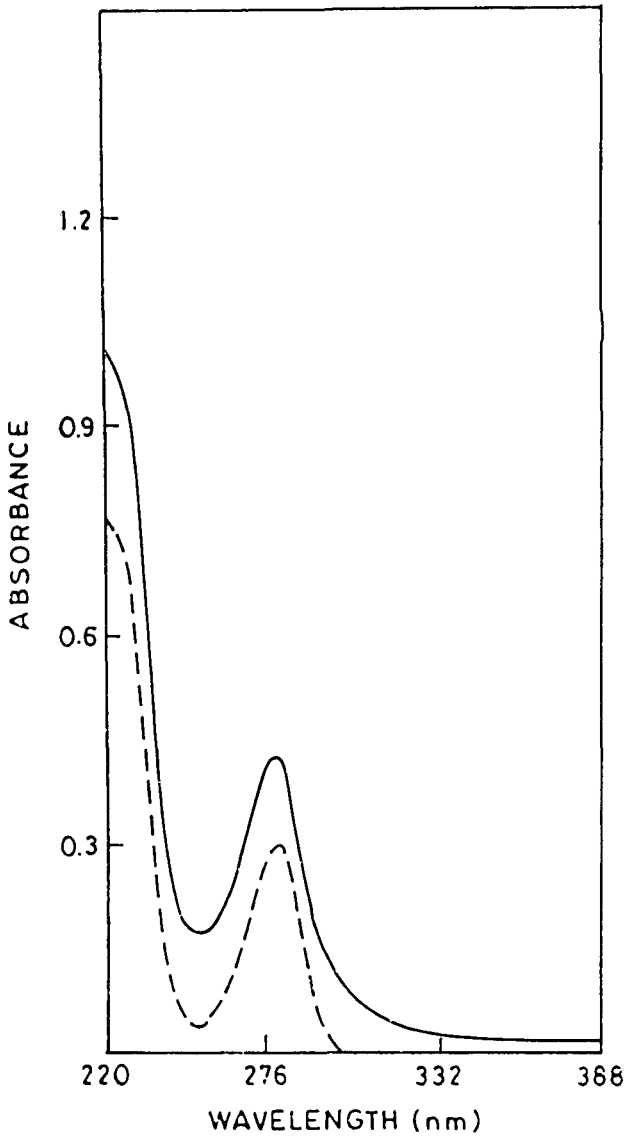
Spectra of solutions containing 0.1mM compound in 10mM Tris-HCl (pH 7.5) were recorded.

(____) - at 0 time

(_ _ _) - after 8 hours of incubation


1.2
0.9
0.6
0.3
ABSORBANCE
220
276
332
388
WAVELENGTH (nm)

(a)


1.2
0.9
0.6
0.3
ABSORBANCE
220
276
332
388
WAVELENGTH (nm)

(b)

and neocuproine have absorption maxima at 480nm and 450nm respectively (Jaselow and Dawson, 1951; Nebesar, 1961). Under the reaction conditions used, neither Cu(II) nor the compounds interfere with these maxima. Figures 15 and 16 show that both capsaicin as well as dihydrocapsaicin are able to reduce Cu(II) to Cu(I) leading to the formation of respective characteristic complexes with bathocuproine/neocuproine.

Stoichiometry of Cu (II) reduction

To determine the stoichiometry of Cu(II) reduction by capsaicin and dihydrocapsaicin, the experiments shown in figures 17 and 18 were performed. Increasing concentrations of Cu(II) were added to fixed concentration of the compound and the results were plotted as a Job plot of equivalents of Cu(II)/compounds as absorption of bathocuproine-Cu(II) at 480 nm (figure 17) and neocuproine-Cu(II) at 450nm (figure 18). The Job plot (Wong *et al.*, 1984) of absorbance vs. Cu (II)/capsaicin (figure18) shows maximum absorbance at a ratio of 3 for both neocuproine and bathocuproine. For dihydrocapsaicin, the maximum absorbance was at a ratio of 2. This implies a 3:1 stoichiometry for the reduction of Cu (II) by free capsaicin and 2:1 by free dihydrocapsaicin.

Fig 15. Detection of capsaicin / dihydrocapsaicin induced Cu(I) production using bathocuproine.

The concentrations of capsaicin, dihydrocapsaicin and Cu(II) were 0.02mM while that of bathocuproine was 0.4mM.

(_____) - bathocuproine + 0.05 mM Cu(I)

(_ _ _ _) - bathocuproine + Cu(II) + capsaicin

(_ . _ . _) - bathocuproine + Cu(II) + dihydrocapsaicin

(_ .... _) - bathocuproine + Cu(II)

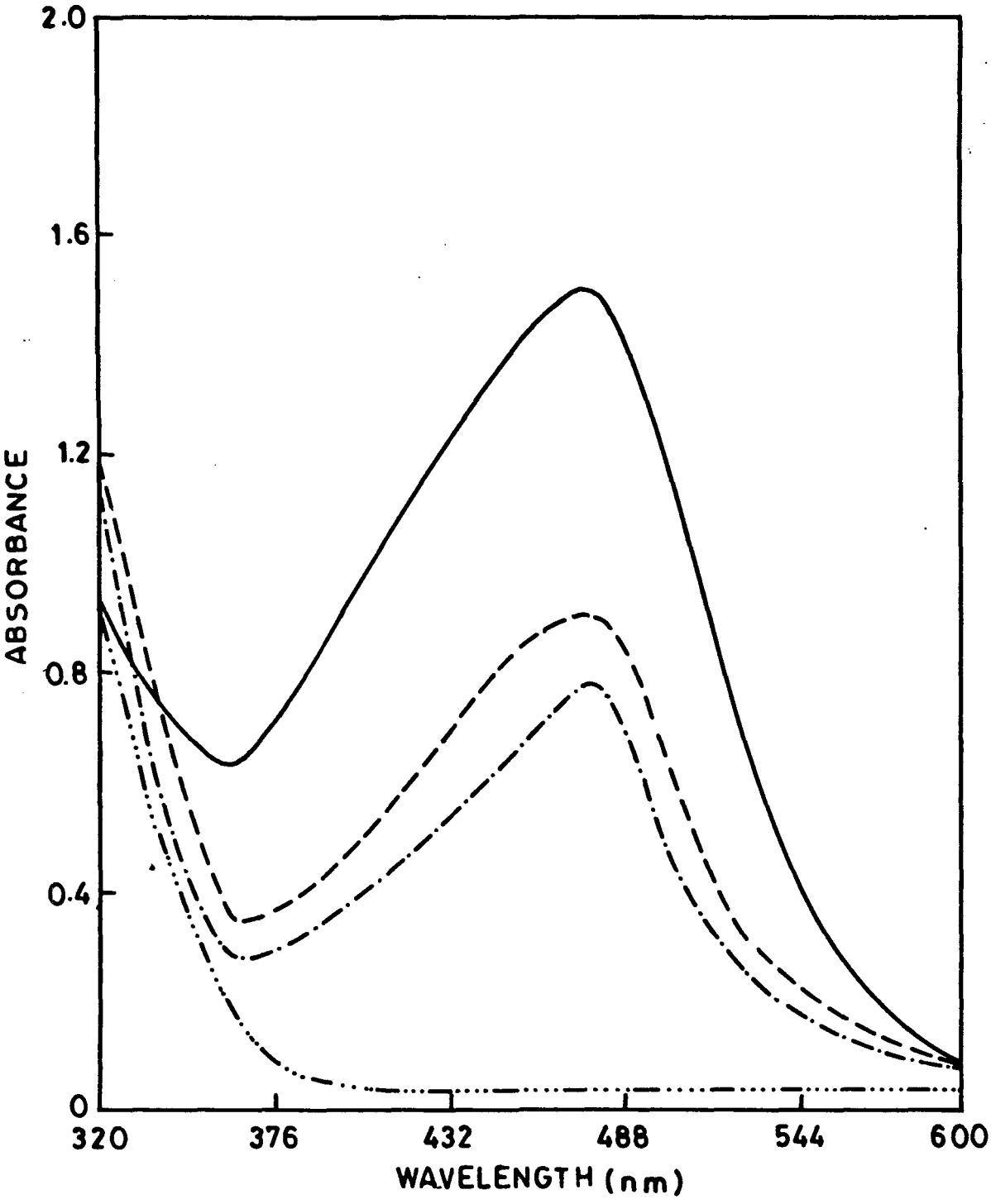

2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE
320
376
432
488
544
600
WAVELENGTH (nm)

Fig 16. Detection of capsaicin / dihydrocapsaicin induced Cu(I) production using neocuproine.

The concentrations of capsaicin, dihydrocapsaicin and Cu(II) were 0.02mM while that of neocuproine was 0.4mM.

(_____) - neocuproine + 0.05 mM Cu(I)

(_ ... _) - neocuproine + Cu(II) + capsaicin

(_ . _ . _) - neocuproine + Cu(II) + dihydrocapsaicin

(_ _ _ _) - neocuproine + Cu(II)

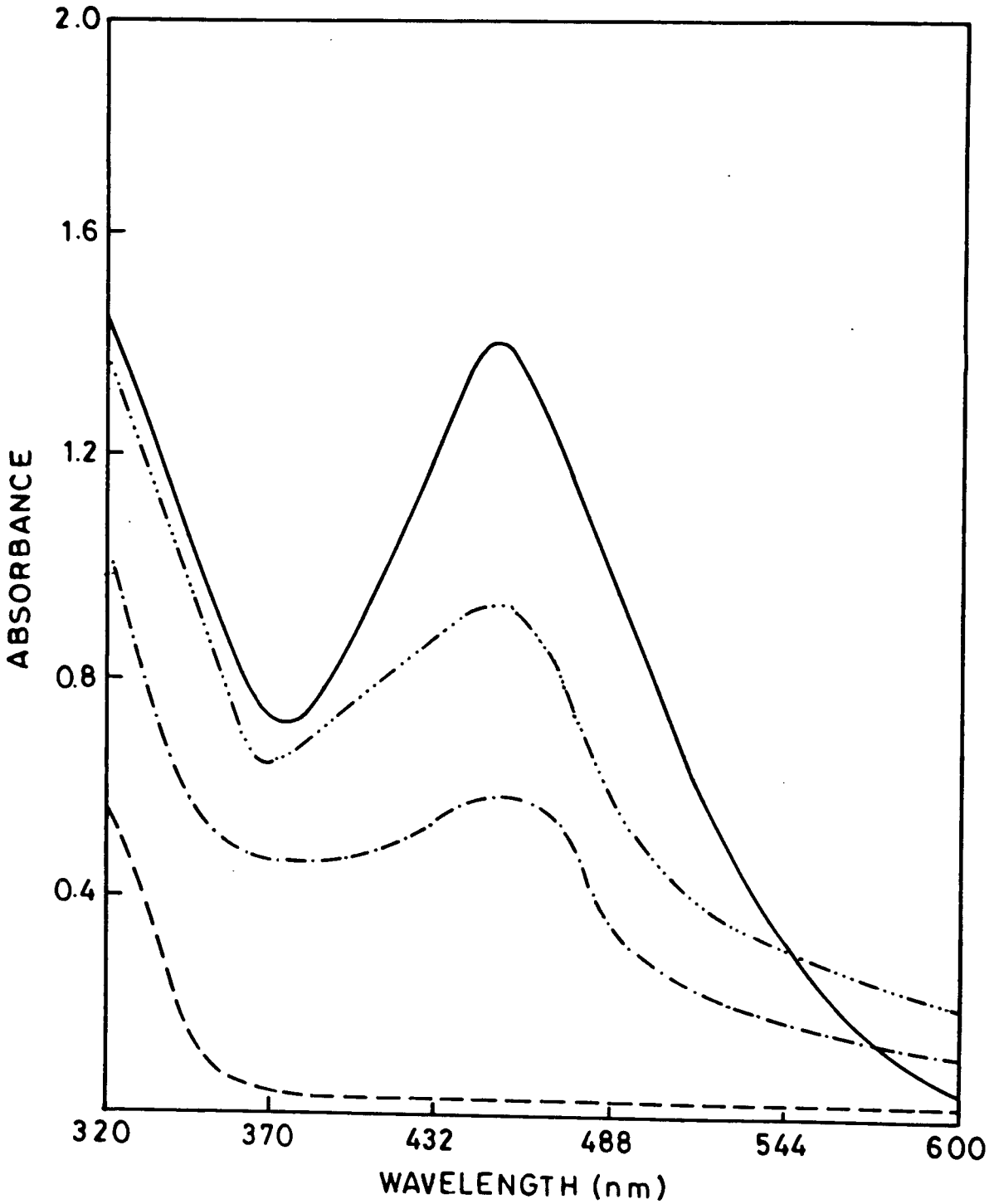
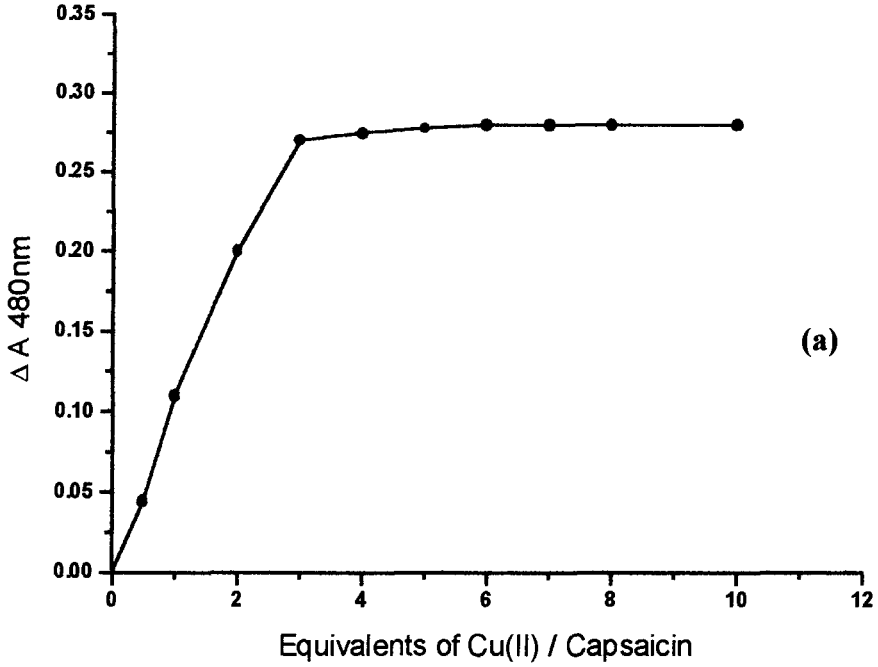

2.0
1.6
1.2
0.8
0.4
ABSORBANCE
320
370
432
488
544
600
WAVELENGTH (nm)

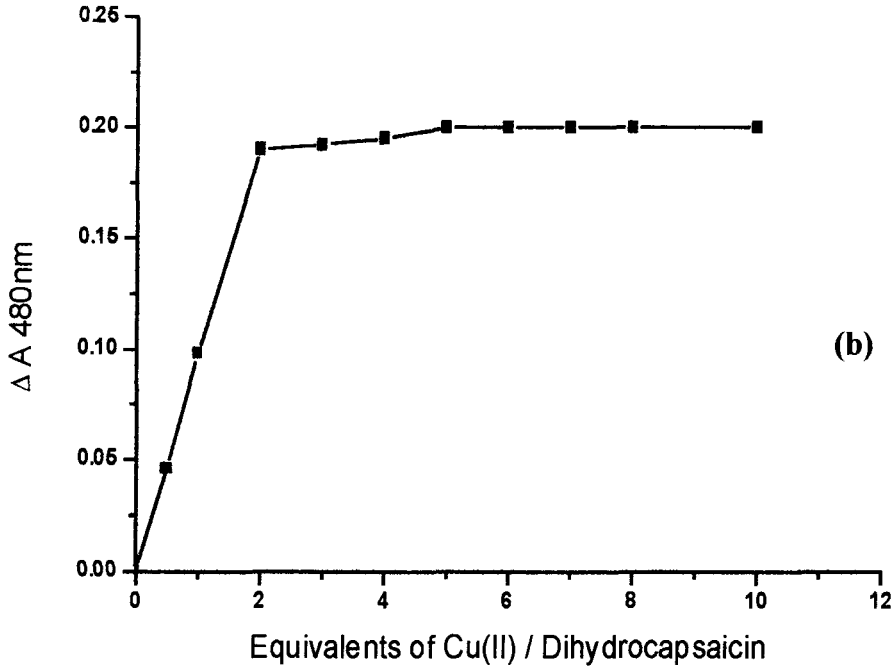
Fig 17. Determination of stoichiometry of the compounds and Cu(II) reaction.

The difference in absorbance at 480 nm of samples with and without added Cu (II) is plotted versus molar equivalents of Cu (II) per molar equivalent of compound. The compound concentration was 0.005 mM in presence of 0.25 mM of bathocuproine. The value of independent variable at the intersection of the two lines is a measure of the moles of Cu (II) converted to Cu (I) per mole of the compound. The values obtained are:

(a) Capsaicin 3:1 and (b) DHC 2:1


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Δ A 480nm
0
2
4
6
8
10
12
Equivalents of Cu(II) / Capsaicin

(a)

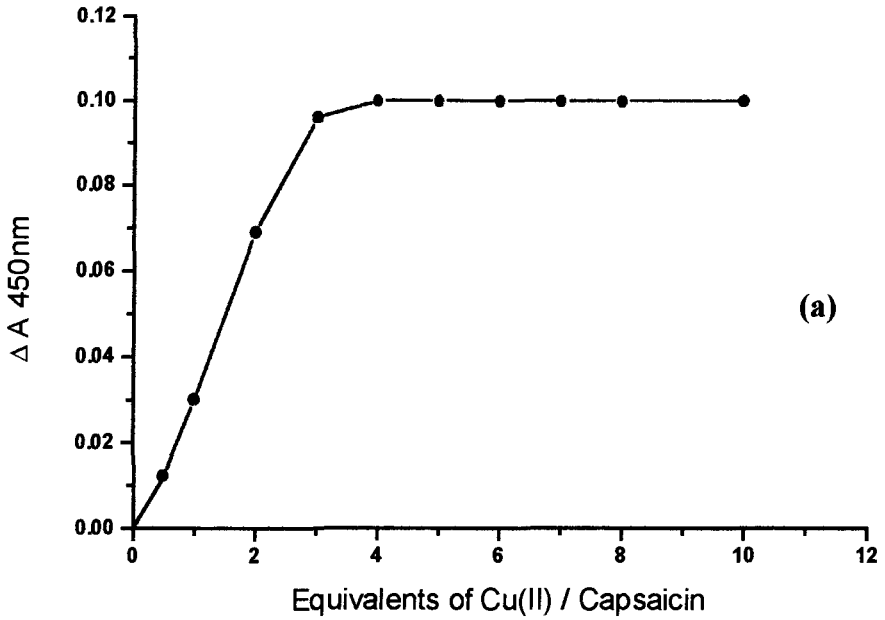

0.25
0.20
0.15
0.10
0.05
0.00
Δ A 480nm
0
2
4
6
8
10
12
Equivalents of Cu(II) / Dihydrocapsaicin

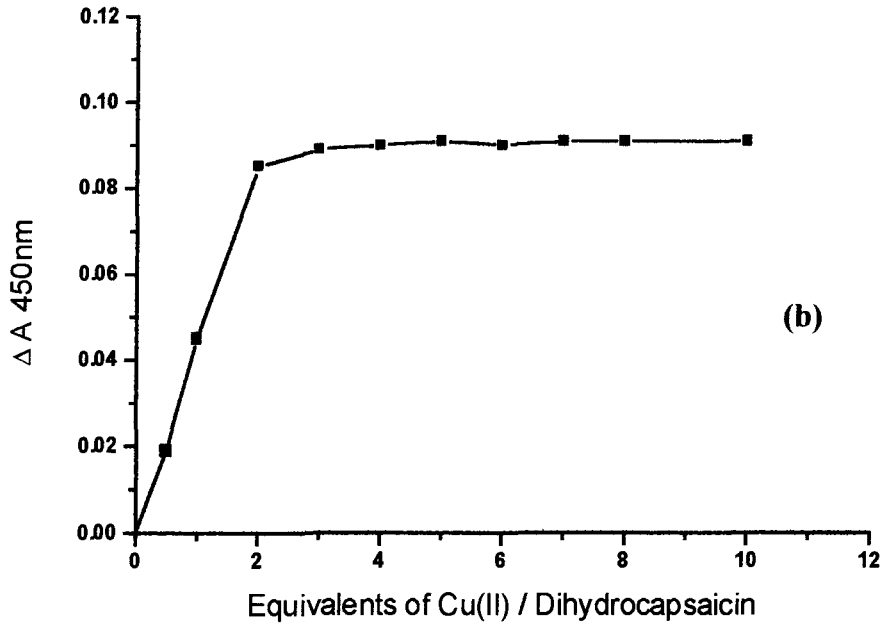
(b)

Fig 18. Determination of stoichiometry of the compounds and Cu(II) reaction.

The difference in absorbance at 450 nm of samples with and without added Cu(II) is plotted versus molar equivalents of Cu (II) per molar equivalent of compound. The compound concentration was 0.005 mM in presence of 0.25 mM of neocuproine. The value of independent variable at the intersection of the two lines is a measure of the moles of Cu (II) converted to Cu (I) per mole of the compound. The values obtained are:

(a) Capsaicin 3:1 and (b) DHC 2:1


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Δ A 450nm
0
2
4
6
8
10
12
Equivalents of Cu(II) / Capsaicin
(a)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Δ A 450nm
0
2
4
6
8
10
12
Equivalents of Cu(II) / Dihydrocapsaicin
(b)

Effect of free radical scavengers on calf thymus DNA breakage by capsaicin/dihydrocapsaicin

Several mutagens of dietary importance such as flavonoids (Rahman *et al.*, 1989) and kojic acid (Bhat and Hadi, 1992) and anticancer drugs such as bleomycin (Sugimura, 1979 ; Ehrenfeld *et al.*, 1987), adriamycin (Elliot *et al.*, 1984) and various other drugs have been shown to degrade DNA in the presence of a metal ion and molecular oxygen. In all these reactions active oxygen species such as hydroxyl radical ($OH^{\cdot}$) and/or singlet oxygen ($^1O_2$) were shown to be the proximal DNA cleaving agents. For this purpose the effect of several free radical scavengers on DNA degradation by capsaicin and dihydrocapsaicin in the presence of Cu(II) was examined. Sodium azide is a singlet oxygen scavenger while superoxide dismutase (McCord and Fridovich, 1969) and catalase remove superoxide anion and hydrogen peroxide respectively. Thiourea and sodium benzoate scavenge hydroxyl radicals. Table I shows the effects of various scavengers on the DNA breakage reaction by capsaicin and dihydrocapsaicin. All the scavengers tested showed varying degrees of inhibition indicating the essential role of oxygen free radicals. It may be noted that thiourea causes maximum inhibition of DNA breakage induced by both capsaicin and dihydrocapsaicin (~83 and 82% respectively). In the case of sodium azide, the inhibition of capsaicin induced DNA damage is 62% while that of dihydrocapsaicin induced DNA damage

Table I : Percent inhibition of S_1 nuclease hydrolysis of calf thymus DNA after treatment with Capsaicin/Dihydrocapsaicin and $CuCl_2$ in the presence of scavengers of reactive oxygen species

Scavengers	Capsaicin		Dihydrocapsaicin	
	DNA Hydrolysed (%)	% Inhibition	DNA Hydrolysed (%)	% Inhibition
Cap/DHC-Cu(II) (Control)	22.37	0	12.33	0
Control + Sodium Azide (50mM)	8.49	62.07	4.33	64.93
Control + Thiourea (50mM)	3.71	83.41	2.24	81.77
Control + Sodium Benzoate(50mM)	10.41	53.43	6.11	50.41
Control + Catalase (100µg/ml)	18.92	15.40	10.15	17.63
Control + SOD (100µg/ml)	19.44	13.09	10.35	16.02

Concentrations of scavengers shown are final reaction concentrations. The reaction mixtures were incubated for 3 hours at 37°C.

Control : Capsaicin/Dihydrocapsaicin (1mM) + Cu(II) (0.5mM)

SOD : Superoxide dismutase

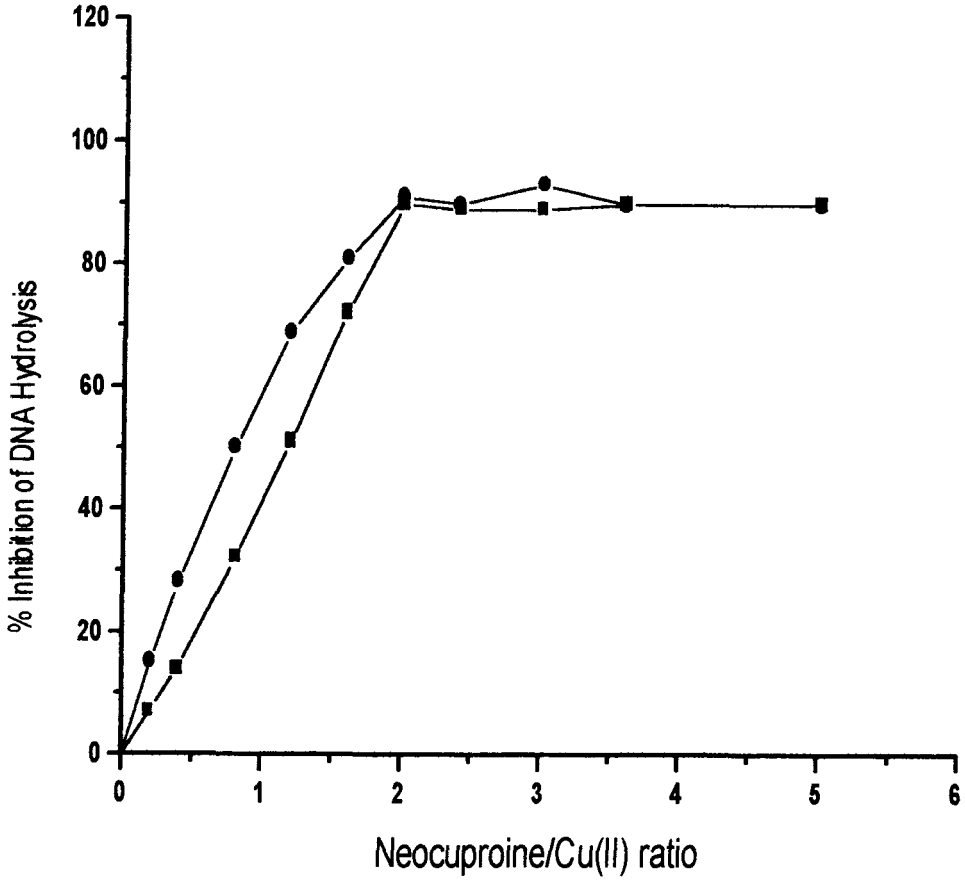
is 65%. These results indicate that in the degradation of DNA by both compounds, a major role is played by hydroxyl radicals and singlet oxygen.

Inhibition of capsaicin/dihydrocapsaicin-Cu(II) induced DNA breakage by neocuproine

It was of interest to determine whether the production of Cu(I) during capsaicin-Cu(II) interaction was necessary for DNA breakage. For this purpose, the Cu(I) specific chelating agent, neocuproine was added to the reaction mixture. Neocuproine forms a stable complex with Cu(I) in aqueous solutions. Previous work in this laboratory (Rahman *et al.*, 1989; Bhat and Hadi, 1992) and others (Que *et al.*, 1980) has confirmed (data not shown) that the neocuproine-Cu(I) complex does not hydrolyze DNA. The inhibition of DNA breakage was examined by using a single ratio of compound and Cu(II) and varying amounts of neocuproine. When increasing amounts of neocuproine were added, there was a progressive decrease in the S_1 nuclease susceptibility of DNA. Results in figure 19 indicate that the percent inhibition is a function of the ratio of neocuproine/Cu(II). The percent inhibition plateaued at a ratio of 2 regardless of the ratio of capsaicin/dihydrocapsaicin and Cu(II) in the reaction mixture. This confirms that Cu(I) is an essential intermediate in the reaction. Cu(II) alone upto a concentration of 1 mM does not cause breakage of DNA (data not shown). This was determined by incubating Cu(II) with DNA and subjecting it to S_1-nuclease hydrolysis.

Fig 19. Effect of increasing neocuproine concentrations on calf thymus DNA degradation by capsaicin /dihydrocapsaicin-Cu(II).

The reaction mixtures were incubated for 6 hours at 37^0C. 0.5mg calf thymus DNA in 10mM Tris-HCl (pH 7.5) was incubated with 1mM capsaicin (●) / dihydrocapsaicin (■) in presence of 0.5mM Cu(II).


120
100
80
60
40
20
0
% Inhibition of DNA Hydrolysis
0
1
2
3
4
5
6
Neocuproine/Cu(II) ratio

Generation of oxygen free radicals by capsaicin/dihydrocapsaicin

a) Generation of hydroxyl radicals

The capacity of capsaicin and dihydrocapsaicin to generate hydroxyl radicals in the presence of Cu(II) was compared. This assay uses deoxyribose as a reporter molecule (Quinlan and Gutteridge, 1987). Hydroxyl radicals react with deoxyribose to form a free radical intermediate that decomposes to form an aldehyde which in turn gives an adduct with TBA. The inhibition of the adduct formation by a quencher of hydroxyl radicals such as thiourea indicates that the assay genuinely measures hydroxyl radical (Table II) increasing concentration of both the compounds leads to a progressively increased formation of the hydroxyl radicals. However, at all concentrations tested, the formation of TBA reactive material was greater in the case of capsaicin indicating that capsaicin is relatively more efficient than dihydrocapsaicin in generating the hydroxyl radicals.

b) Generation of hydrogen peroxide

The method involves the oxidation of titanium to pertitanic acid by hydrogen peroxide (Hozumi, 1969). Table III shows the production of hydrogen peroxide with increasing concentrations of the compounds. It is seen that hydrogen peroxide formation increases with increasing concentrations of both capsaicin and

Table II : Formation of hydroxyl radical as a function of Capsaicin/Dihydrocapsaicin concentration

Compound (μM)	TBARS (nmoles) generated by	
	Capsaicin	Dihydrocapsaicin
50	0.55	0.40
100	0.76	0.49
200	0.93	0.58
300	0.99	0.61
400	1.22	0.76
400 + Thiourea (50mM)	0.51	0.43

Reaction conditions are as described in 'Methods'. The concentrations of Capsaicin and Dihydrocapsaicin are final reaction concentrations. The final Cu(II) concentration was 400μM.

Table III : Generation of H_2O_2 by Capsaicin and Dihydrocapsaicin

Compound (μmoles)	nmoles of H_2O_2	
	Capsaicin	Dihydrocapsaicin
0.2	0.04	-
0.4	0.12	-
1.2	0.29	0.04
2.0	0.31	0.043
4.0	0.33	0.13
4.0 + Catalase (0.1mg/ml)	0.053	0.023

The H_2O_2 was produced as a function of increasing concentration of compound. Detailed procedure is described in 'Methods'.

dihydrocapsaicin. In the presence of catalase, the production of hydrogen peroxide is inhibited confirming that the procedure employed genuinely measures the peroxide. Also, as in the case of hydroxyl radical, capsaicin is a more efficient generator of hydrogen peroxide.

Spectra of compounds with increasing Cu(II)

Figure 20 shows the effect of increasing Cu(II) concentration on the intensity of the absorption of spectra of capsaicin (a) and dihydrocapsaicin (b). As can be seen, there is a progressive increase in the intensities of the peak absorbing at 277 nm of both capsaicin and dihydrocapsaicin. The reason for progressive increase in absorption can not be ascertained. However, the results do suggest that copper is able to bind to the two compounds.

Protein fragmentation by capsaicin/dihydrocapsaicin in presence of Cu(II)

Previous studies in this laboratory had shown that quercetin-Cu(II) system which cleaves DNA is also capable of protein fragmentation. It was therefore of interest to examine whether the capsaicin-Cu(II) system also possesses the same property. Figure 21 shows the extent of protein fragmentation by increasing concentration of capsaicin/dihydrocapsaicin in the presence of a fixed concentration of Cu(II). The method determines the formation of acid soluble

Fig 20. Effect of increasing Cu(II) concentration on the absorption spectrum of capsaicin(a) and dihydrocapsaicin(b).

(_____) compound alone

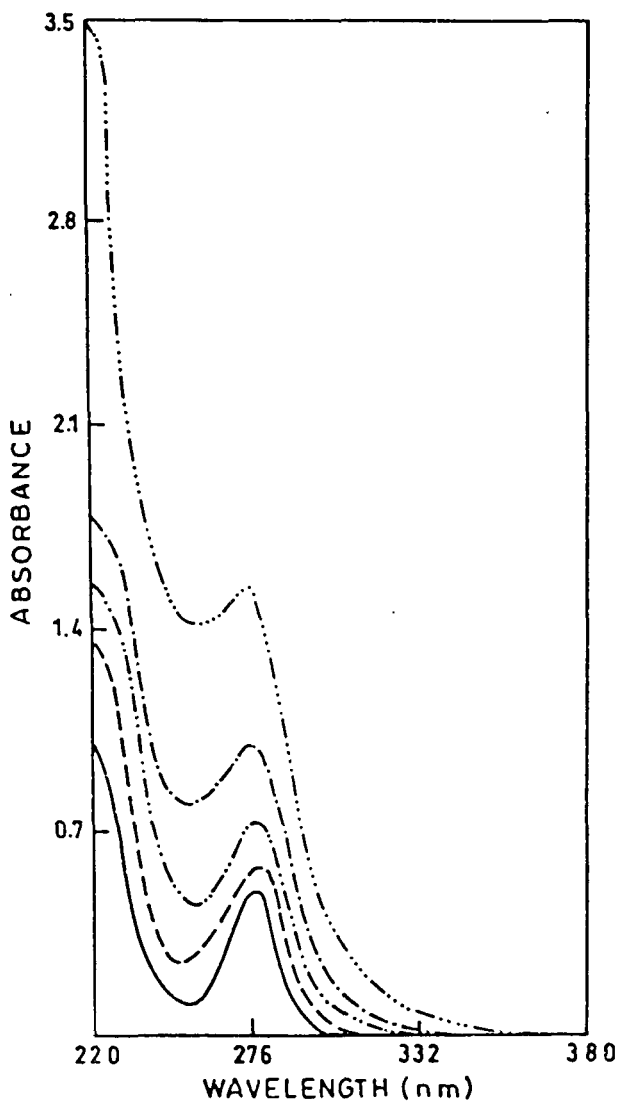
(_ _ _) compound : Cu(II) 1:1

(_ . . _) 1:10

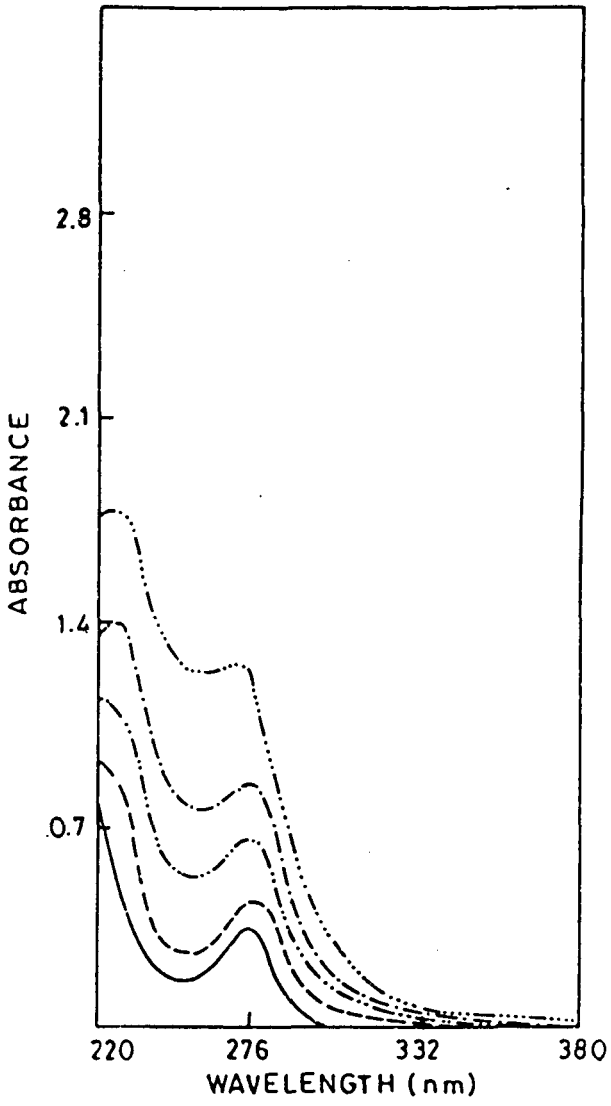
(_ . _) 1:25

(_ ... _) 1: 50

Compound concentration was 0.05mM in 10mM Tris-HCl (pH 7.5).


3.5
2.8
2.1
1.4
0.7
ABSORBANCE
220
276
332
380
WAVELENGTH (nm)

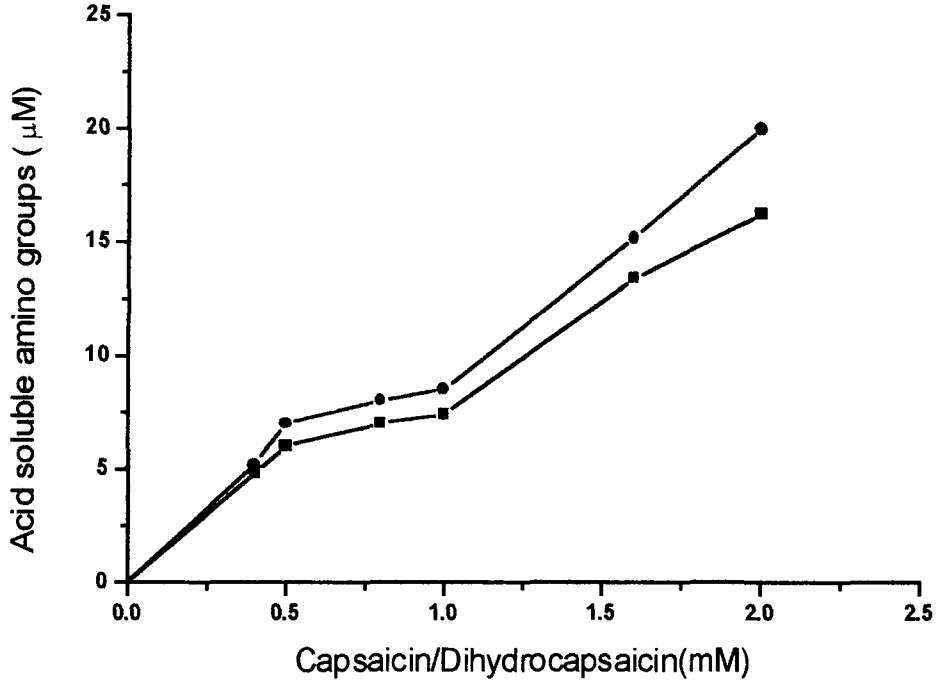
(a)


2.8
2.1
1.4
0.7
ABSORBANCE
220
276
332
380
WAVELENGTH (nm)

(b)

Fig 21. Protein fragmentation by capsaicin / dihydrocapsaicin in the presence of Cu(II).

BSA (2mg/ml) was incubated with increasing concentrations of capsaicin (●) and dihydrocapsaicin (■) in the presence of 1mM Cu(II) for 8 hours at 37^0C. For details, see "Methods".


Acid soluble amino groups (μM)
25
20
15
10
5
0
0.0
0.5
1.0
1.5
2.0
2.5
Capsaicin/Dihydrocapsaicin(mM)

amino groups by TNBS. It is seen that there is a concentration dependent increase in protein fragmentation by both capsaicin and dihydrocapsaicin. Also, the fragmentation induced by concentrations upto 1mM is nearly similar for both capsaicin and dihydrocapsaicin. It is only at concentrations above 1mM that we find capsaicin causing more fragmentation than dihydrocapsaicin. At a 2 mM compound concentration, capsaicin causes about 20% more fragmentation than dihydrocapsaicin.

Binding of capsaicin and dihydrocapsaicin with BSA

Figure 22 (a) and (b) show the change in the fluorescence emission spectrum of BSA in the presence of increasing concentrations of capsaicin/dihydrocapsaicin. When a solution of BSA is excited at 280 nm it exhibits a fluorescence emission spectrum with a maxima at 325 nm. The addition of compounds to BSA causes quenching. Increasing the concentration of the compounds enhances the quenching effect. A comparison of (a) and (b) shows that the extent of quenching induced by BSA-capsaicin ratio of 1:5 is almost similar to that induced by BSA-dihydrocapsaicin ratio of 1:10. This would suggest that the binding of capsaicin has a greater shielding effect on the tryptophan moieties in BSA.

Fig. 22. Effect of increasing concentrations of capsaicin(a) and dihydrocapsaicin(b) on the fluorescence spectra of BSA (1mM).

(a)

(___) BSA alone

(_ . _) BSA : capsaicin 1:1

(_ ... _) 1:5

(b)

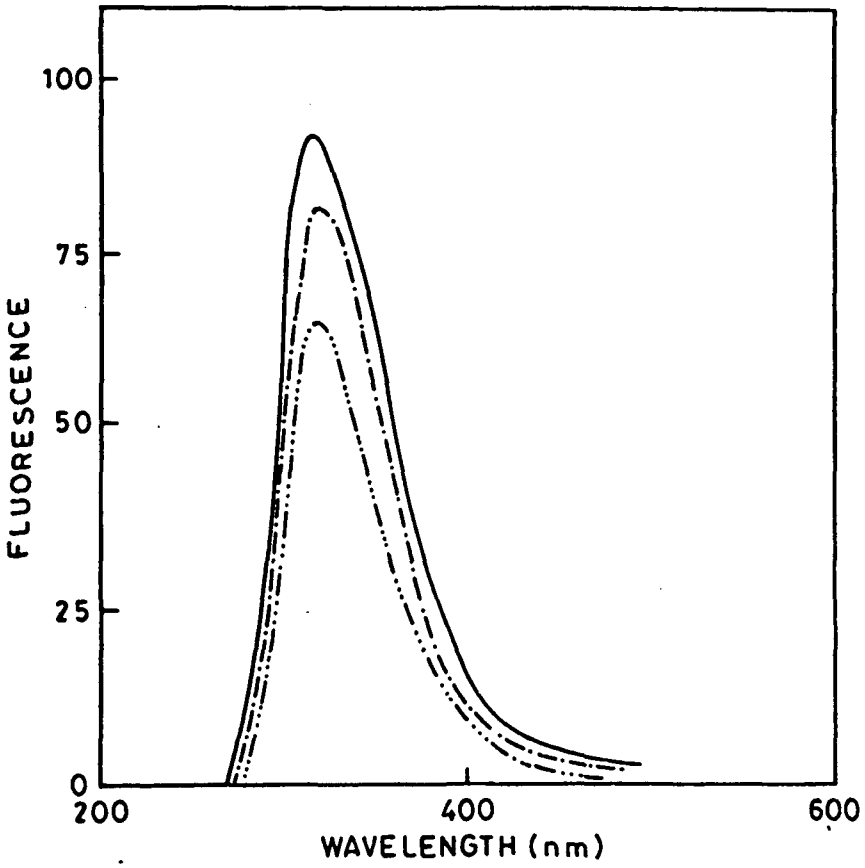
(___) BSA alone

(_ . _) BSA : dihydrocapsaicin 1:1

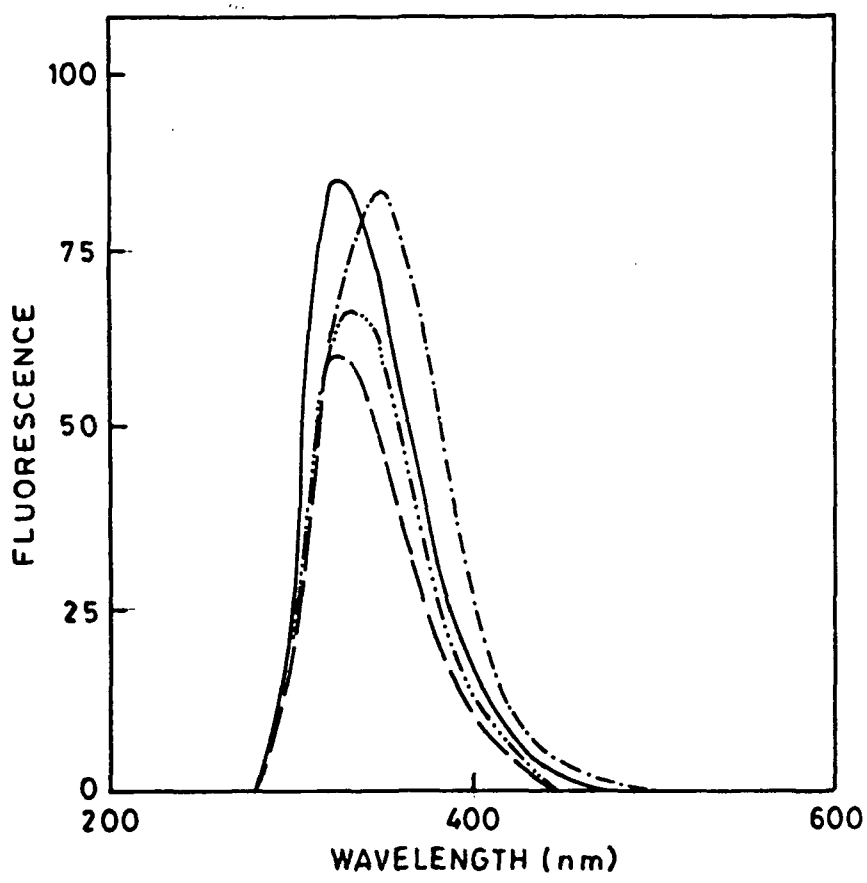
(_ .... _) 1:2

(_ _ _) 1:5

The wavelength of excitation λ_{ex} was 280nm.


100
75
50
25
0
FLUORESCENCE
200
400
600
WAVELENGTH (nm)

(a)


100
75
50
25
0
FLUORESCENCE
200
400
600
WAVELENGTH (nm)

(b)

The formation of complexes involving DNA and compounds

Figure 23 shows that when a solution of capsaicin or dihydrocapsaicin is excited at 288 nm, it exhibits a fluorescence emission spectrum with a maximum around 315 nm. The addition of DNA to compounds (figure 24a and b) results in dose dependent quenching of the fluorescence. Also, both capsaicin and dihydrocapsaicin show a gradual shift in the emission peak as the ratio of compound : base pair is increased. At compound:base pair molar ratio of 1:6, the emission maxima for capsaicin shows a shift to 293 nm from 314 nm. In case of dihydrocapsaicin the maxima also shifts to 293 nm from 316nm. Figure 25 (a) shows that extent of quenching of capsaicin by DNA is more for the native form than the denatured one. However, addition of native and denatured forms of calf thymus DNA to dihydrocapsaicin(b) results in equal amount of quenching of the emission spectra.

Figure 26 shows the effect of sodium chloride on fluorescence quenching of compounds by DNA. With increasing NaCl concentrations, a significant change in the fluorescence quenching of both the compounds is observed. Thus, increased ionic strength seem to affect the interaction of compounds to DNA.

To assess the binding of copper (II) ions to capsaicin/dihydrocapsaicin, the compound solutions were excited at 288 nm in the presence of increasing Cu(II)

Fig 23. Fluorescence emission spectra of 0.05mM capsaicin (___) and dihydrocapsaicin (_ _ _) in 10mM Tris-HCl (pH 7.5).

The wavelength of excitations λ_{ex} is 288nm.

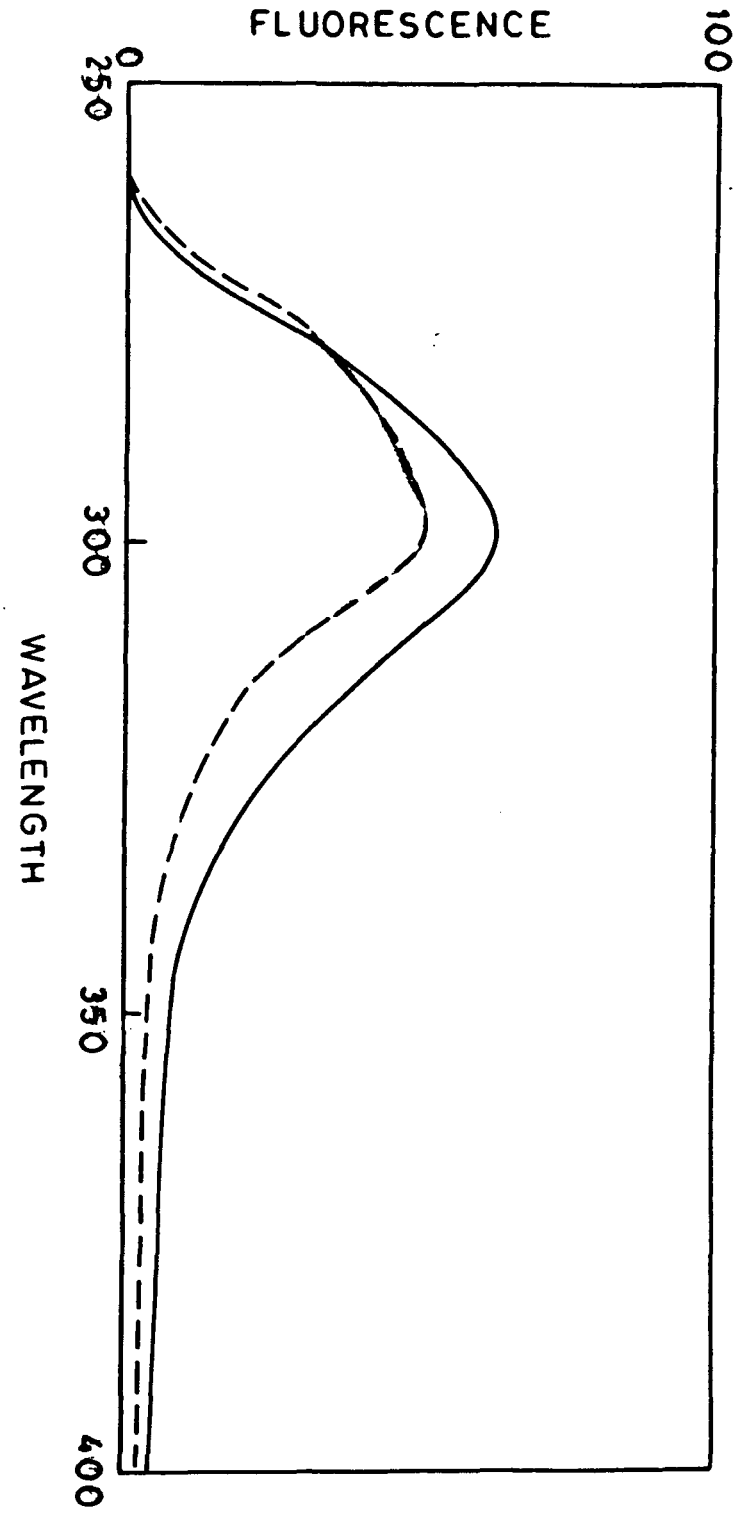

FLUORESCENCE
0
100
250
300
350
400
WAVELENGTH

Fig 24. Effect of increasing native DNA base pairs on the fluorescence emission spectra of capsaicin(a) and dihydrocapsaicin(b).

(a)

(___) capsaicin alone

(_._) capsaicin : DNA ratio 1:0.5

(_ _ _) 1:1

(__ __) 1:2

(.....) 1:4

(_ .... _) 1:6

(b)

(___) dihydrocapsaicin alone

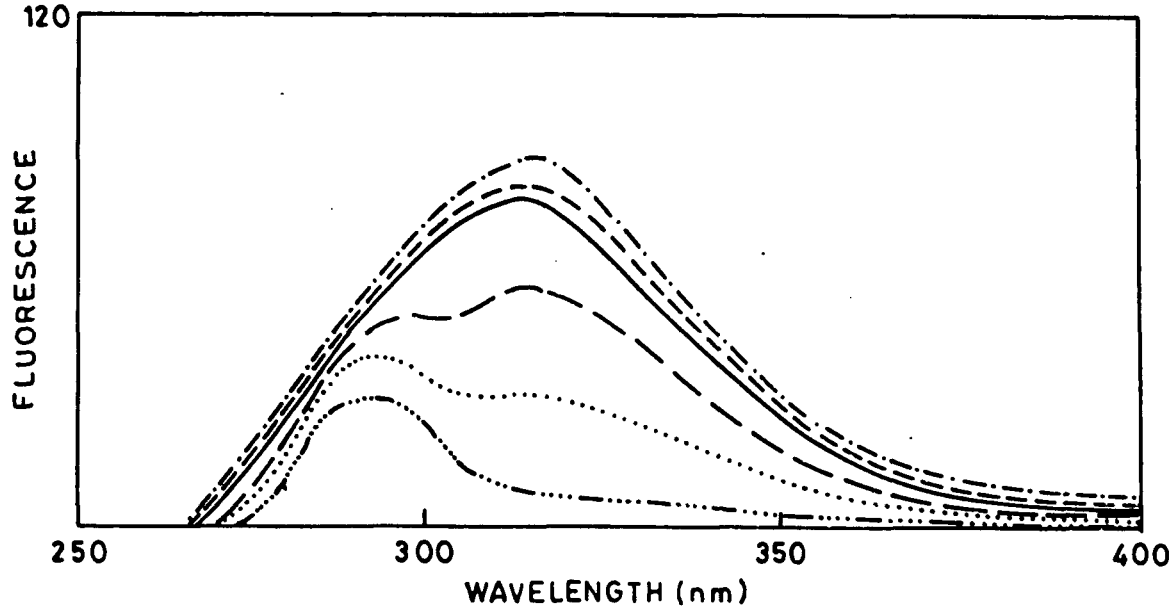
(_ _ _) dihydrocapsaicin : DNA ratio 1:0.5

(_ . _) 1:1

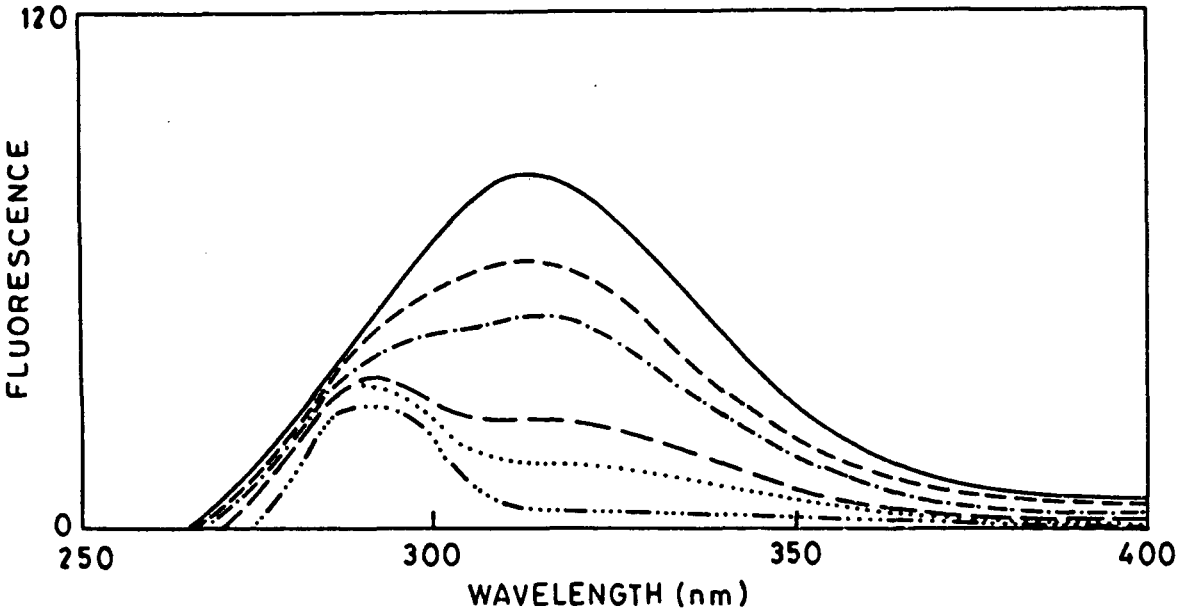
(__ __) 1:2

(.....) 1:4

(_ .... _) 1:6


120
FLUORESCENCE
250
300
350
400
WAVELENGTH (nm)

(a)


120
FLUORESCENCE
0
250
300
350
400
WAVELENGTH (nm)

(b)

Fig 25. Effect of denatured and native DNA on fluorescence emission of capsaicin(a) and dihydrocapsaicin(b).

0.05mM compounds in 10mM Tris-HCl (pH 7.5) were mixed with 0.25mM calf thymus DNA. λ_{ex} was 288nm.

(a)

(___) capsaicin alone

(_ . _) DNA alone

(_ _ _) Native DNA + capsaicin (1:5)

(_ ... _) Denatured DNA + capsaicin (1:5)

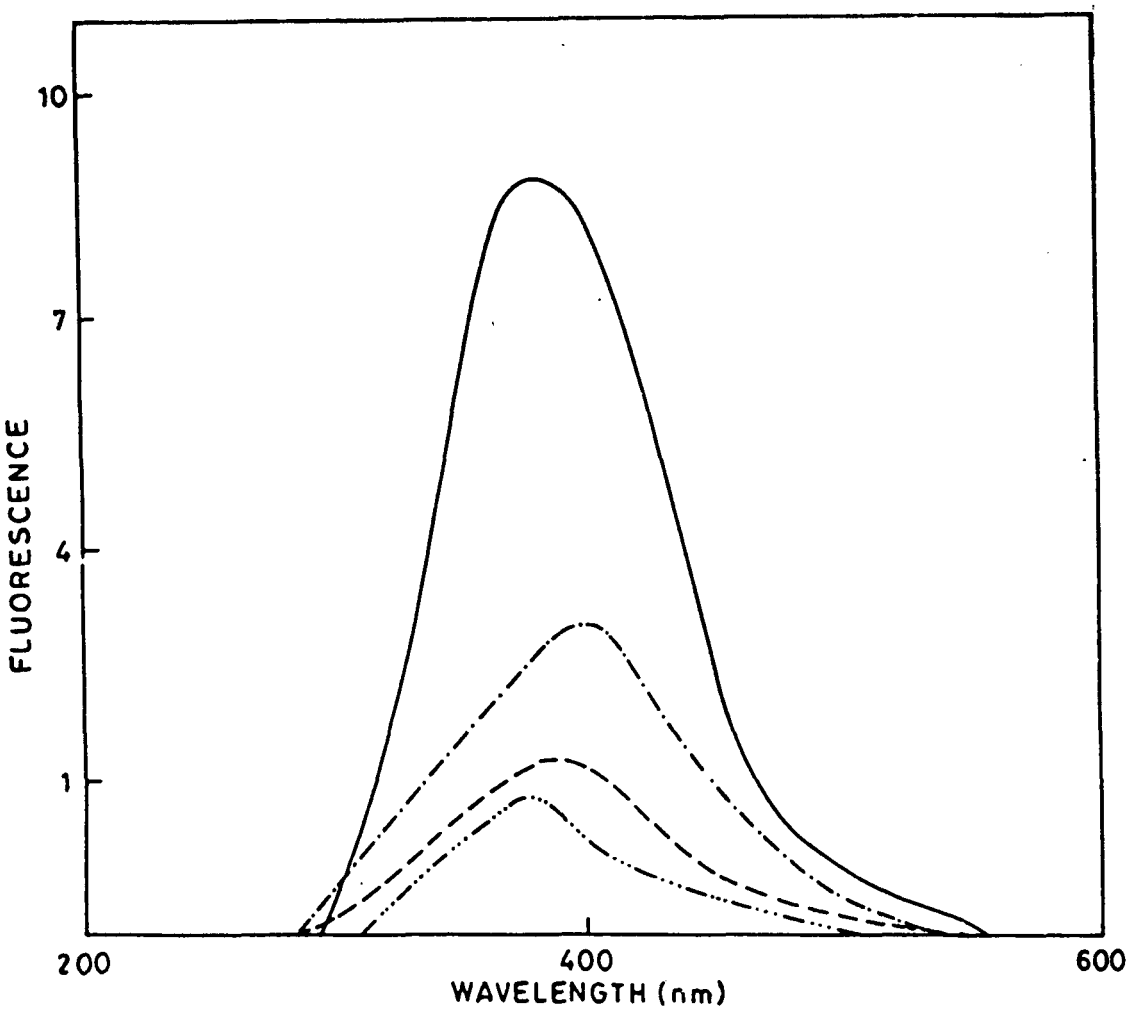
(b)

(___) dihydrocapsaicin alone

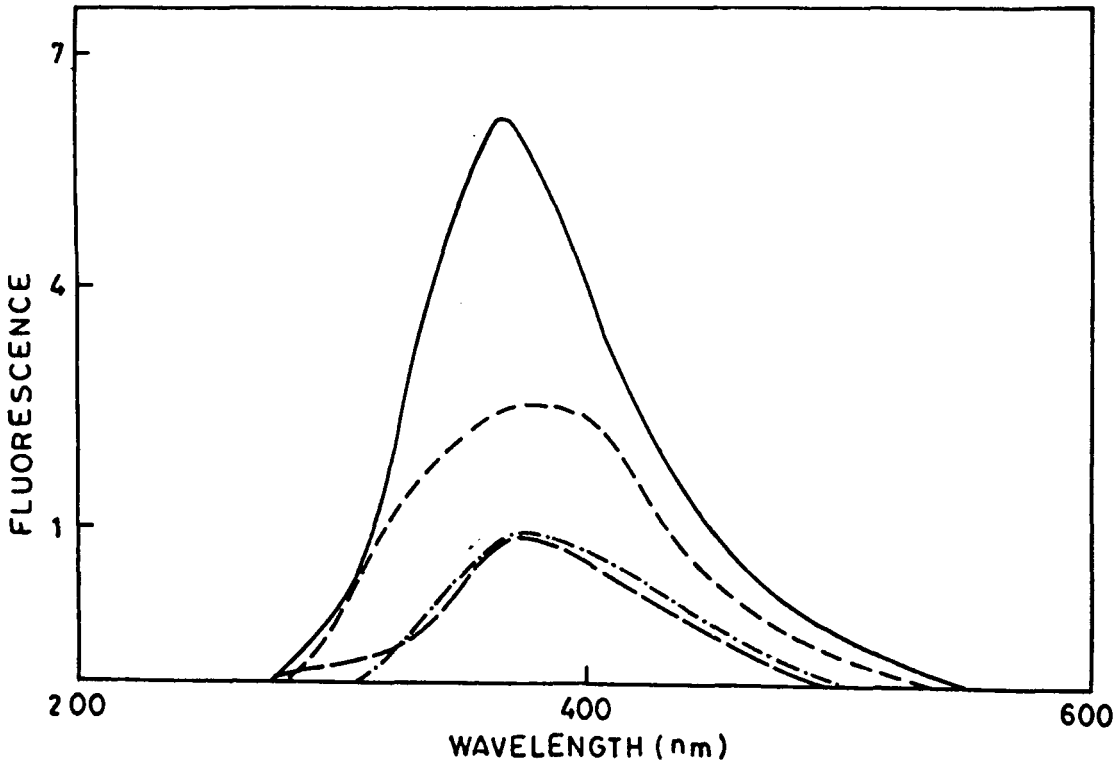
(_ _) DNA alone

(_ . _) Native DNA + dihydrocapsaicin (1:5)

(__ __) Denatured DNA + dihydrocapsaicin (1:5)


10
7
4
1
FLUORESCENCE
200
400
600
WAVELENGTH (nm)

(a)


7
4
1
FLUORESCENCE
200
400
600
WAVELENGTH (nm)

(b)

Fig 26. Changes in the fluorescence emission of capsaicin(a) / dihydrocapsaicin(b)-DNA complex in the presence of sodium chloride.

The concentrations of compounds and DNA were 0.05mM and 0.025mM respectively.

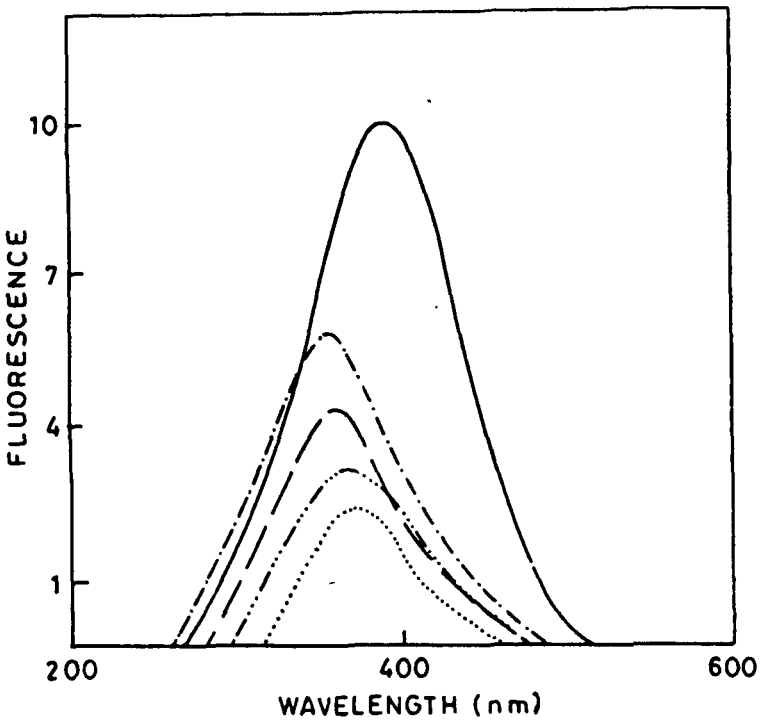
(___) compound alone

(_ . _) compound + DNA + 0.1mM NaCl

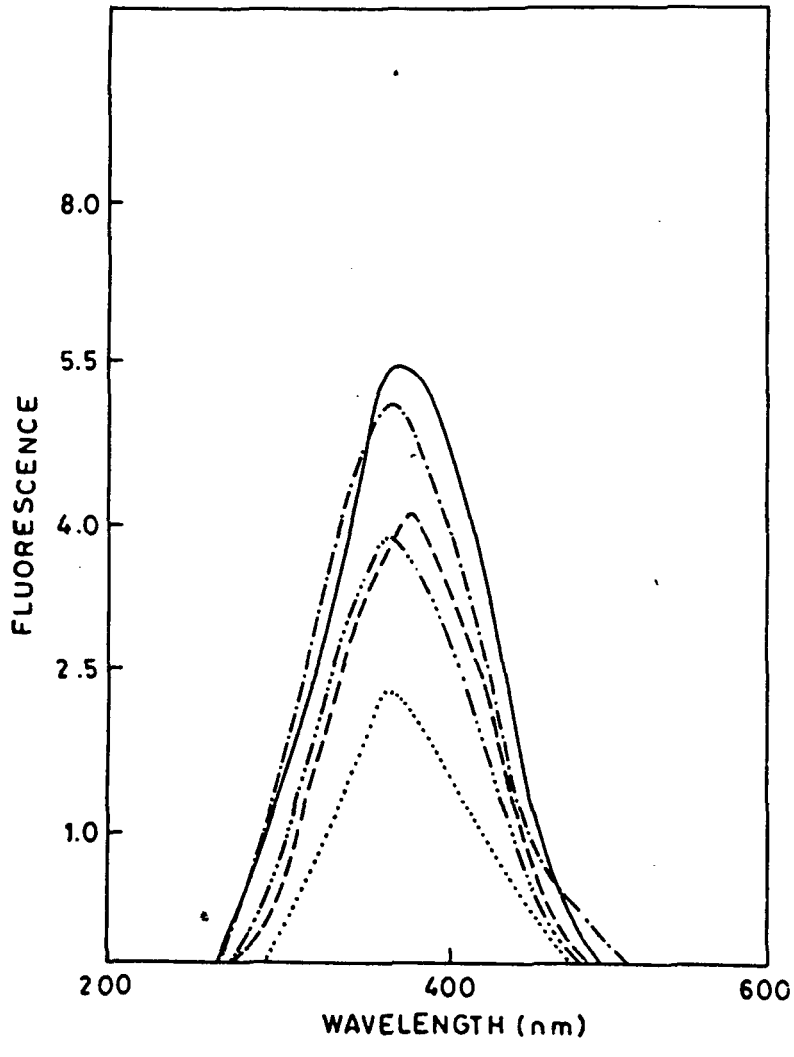
(__ __) compound + DNA + 0.5mM NaCl

(_ ... _) compound + DNA + 1mM NaCl

(…….) native DNA alone


10
7
4
1
FLUORESCENCE
200
400
600
WAVELENGTH (nm)

(a)


8.0
5.5
4.0
2.5
1.0
FLUORESCENCE
200
400
600
WAVELENGTH (nm)

(b)

concentrations (1:0.5, 1:1 and 1:2). Figure 21(a) shows that with increasing Cu(II) concentration the emission maxima of capsaicin shifts from 313 to 293 nm. The shift in maxima of dihydrocapsaicin for similar concentrations is from 313 nm (compound alone) to 286nm (1:2 ratio). The results are indicative of the formation of a novel species presumably a capsaicin-dihydrocapsaicin-Cu(II) complex and are in confirmation of the results obtained by recording the absorption spectrum with copper (figure20).

Inhibition of hydroxyl radical mediated DNA degradation by capsaicin/dihydrocapsaicin

Most studies on capsaicins have centered on the evaluation of their antioxidant action. However, as in the case of other plant derived antioxidants such as flavonoids and tannins in the previous studies, I have shown that capsaicins also act as prooxidants and cause DNA degradation in presence of copper ions. In order to examine the relationship between the prooxidant and antioxidant activity of capsaicins the latter action was also studied. The generation of hydroxyl radical was carried out by reduction of hydrogen peroxide by Fe(II) (Thomas *et al.*, 1986). Table IV quantitates the extent of inhibition offered by increasing concentrations of capsaicin and dihydrocapsaicin (0.05, 0.1 and 0.2 mM respectively). A concentration dependent increase in the extent of inhibition is seen. It is also seen

Fig 27. Effect of increasing compound-Cu(II) ratio on fluorescence emission of capsaicin(a) and dihydrocapsaicin(b).

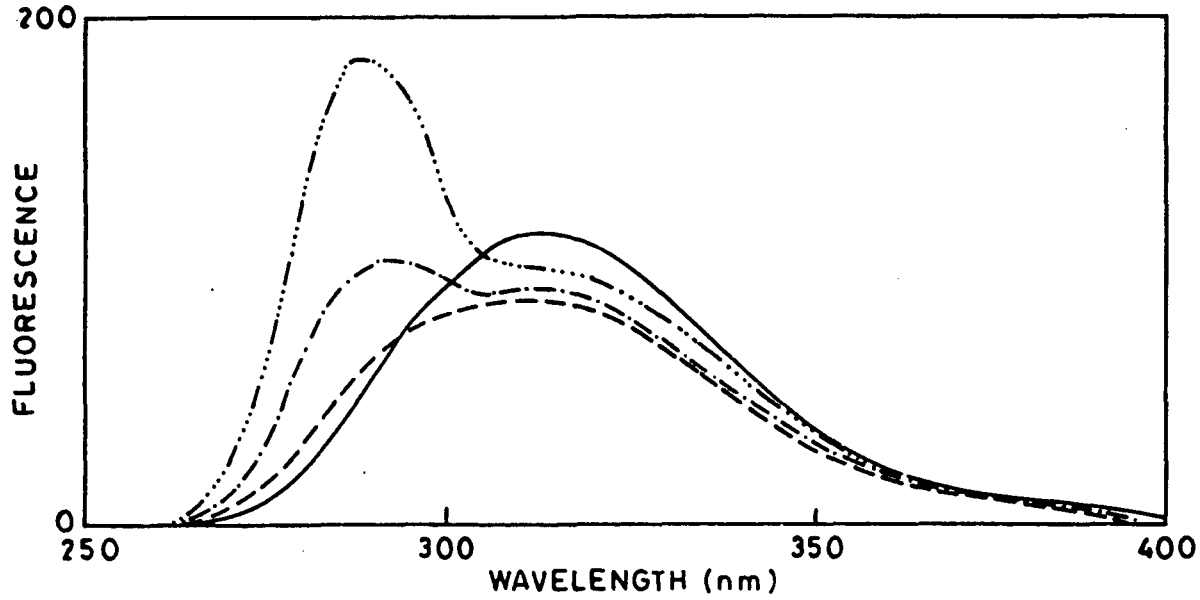
0.05mM of compound in 10mM Tris-HCl (pH 7.5) was mixed with increasing concentrations of Cu(II). The wavelength of excitation was 288nm.

(___) compound alone

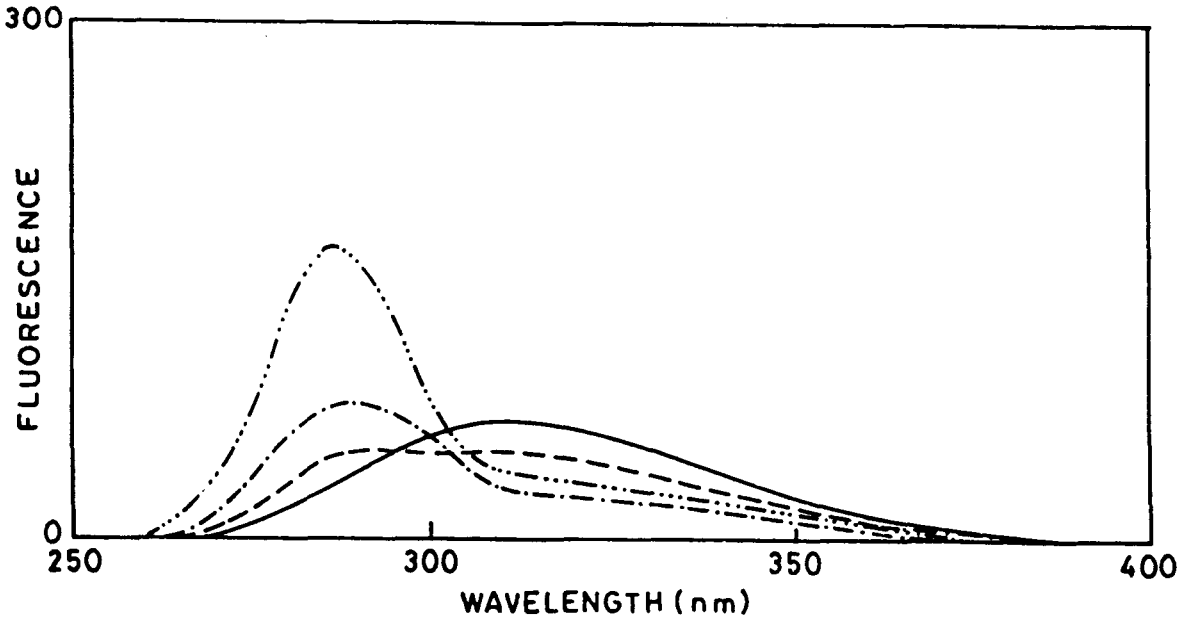
(_ _ _) compound + Cu(II) 1:0.5

(_ . _) compound + Cu(II) 1:1

(_ .... _) compound + Cu(II) 1:2


200
FLUORESCENCE
0
250
300
350
400
WAVELENGTH (nm)

(a)


300
FLUORESCENCE
0
250
300
350
400
WAVELENGTH (nm)

(b)

Table IV : Percent inhibition of S_1 nuclease hydrolysis of calf thymus DNA after treatment with Fe(II)-EDTA (Fenton's reagent) in the presence of different concentrations of Capsaicin (Cap) and Dihydrocapsaicin (DHC).

	DNA Hydrolysed (%)	% Inhibition
Control	50.00	0
Control + Cap (0.05mM)	42.30	15.40
Control + Cap (0.1mM)	34.41	31.18
Control + Cap. (0.2mM)	19.84	60.40
Control + DHC (0.05mM)	44.38	11.24
Control + DHC (0.1mM)	39.96	20.08
Control + DHC (0.2mM)	29.45	41.10

Capsaicin and Dihydrocapsaicin concentrations shown are their final reaction concentrations. Control has 0.5mg calf thymus DNA in 10mM Tris-HCl (pH 7.5) incubated with 1mM Sodium Ascorbate (pH 7.5), 0.03% H_2O_2 and Fe-EDTA for 2 hours at 37^0C.

that capsaicin is a stronger inhibitor of DNA cleavage, as at all concentrations tested it results in a greater inhibition of DNA degradation than dihydrocapsaicin.

To further substantiate these results the conversion of supercoiled plasmid to relaxed and linear forms by hydroxyl radicals was studied in the presence of similar compound concentrations. As seen in figure 28, lane 2, supercoiled plasmid DNA is degraded by Fe(II)-EDTA in presence of H_2O_2 and ascorbate. As evidenced by the relative intensity of the linear bands, capsaicin (lanes 3-5) shows progressively increasing protection while dihydrocapsaicin (lanes 6-8) is not as effective. The result corroborates the findings of the previous experiments where the S_1 susceptibility of calf thymus DNA damaged by the hydroxyl radicals was examined.

Inhibition of L-DOPA-Cu(II) mediated degradation of plasmid DNA

As already explained in the first chapter, the endogenous metabolite L-DOPA causes strand breaks in DNA in the presence of copper ions (Husain and Hadi, 1995). In order to examine whether such DNA breakage is also inhibited by capsaicins, the experiment shown in figure 29 was carried out. As seen in lane 2 of the figure, supercoiled plasmid DNA is completely degraded by DOPA-Cu(II). The effect of increasing concentrations of dihydrocapsaicin (lanes 3-5) and capsaicin (lanes 6-8) is also shown. It is evident that compared to dihydrocapsaicin, capsaicin

Fig 28. Inhibition of hydroxyl radical mediated pBR322 DNA degradation by capsaicin / dihydrocapsaicin.

The system used for the generation of hydroxyl radical contains H_2O_2 (3.75 X 10^{-3}%), sodium ascorbate (0.125mM) and Fe(II)-EDTA (2.5μM Fe(II)-5μM EDTA). A stock solution of Fe(II)-EDTA was prepared immediately before use by mixing equal volumes of freshly prepared 0.4mM Fe(II) [by dissolution of $(NH_4)Fe(SO_4)_2.6H_2O$] and 0.8mM EDTA. DNA in 10mM Tris-HCl (pH 7.5) was incubated with hydroxyl radical generating system (lane2), in presence of 0.1, 0.2 and 0.4mM of capsaicin and dihydrocapsaicin (lanes 3,4,5 and 6,7,8 respectively). Reaction mixture was incubated at 37°C for 30 minutes. Lane 1: DNA alone.

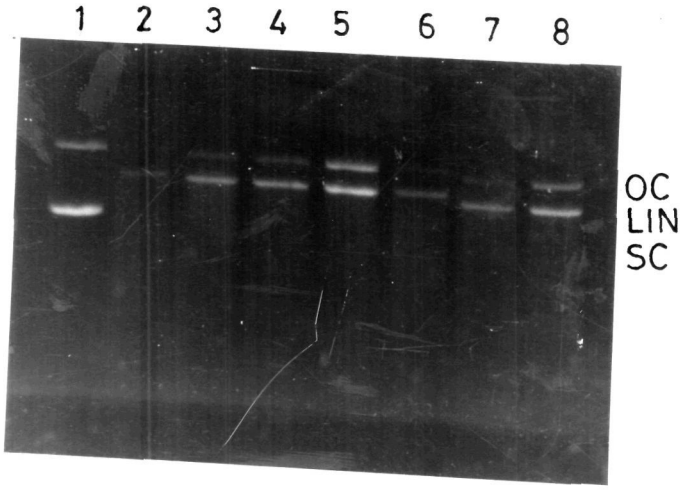
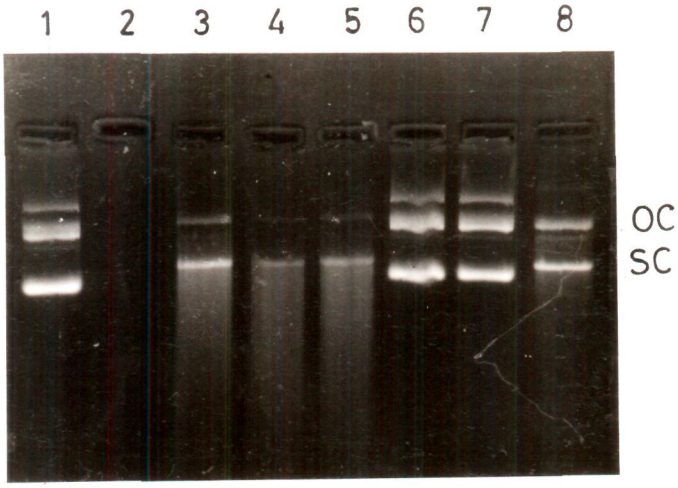

1 2 3 4 5 6 7 8
OC
LIN
SC

Fig 29. Effect of capsaicin/dihydrocapsaicin on L-DOPA-Cu(II) mediated degradation of pBR322 DNA.

Lane 1: DNA alone, lane 2: DNA + DOPA + Cu(II), lanes 3-5: 25, 50 and 100μM dihydrocapsaicin and lanes 6-8: 25,50 and 100μM capsaicin. L-DOPA and Cu(II) were 50μM each. The reaction mixture was incubated for 45 minutes at room temperature.


1
2
3
4
5
6
7
8
OC
SC

offers a greater degree of protection as evidenced by the presence of supercoiled and relaxed circular DNA whereas in the case of dihydrocapsaicin these molecules are completely degraded.

Effect of capsaicin/dihydrocapsaicin on production of singlet oxygen ($^1O_2$) by riboflavin.

Riboflavin is a known producer of singlet oxygen (Naseem *et al.*, 1988 ; Joshi, 1985). Its production was determined in aqueous solution by the bleaching of p-nitroso-dimethyl aniline (pRNO) as described by Kralijic and Mohsini (1978). When sodium azide, a scavenger of singlet oxygen was added to the reaction mixture the bleaching of pRNO decreased substantially, confirming the production of singlet oxygen by riboflavin. A decrease in the bleaching of pRNO was observed when capsaicin and dihydrocapsaicin were included in the reaction mixture, thus indicating the scavenging of singlet oxygen, albeit to different extents (Figure 30). It is also evident that capsaicin is a better scavenger of singlet oxygen than its saturated analog dihydrocapsaicin. Also, a comparison of the concentrations of sodium azide and capsaicin (2 mM and 0.2 mM respectively) with the extent of inhibition of bleaching indicates that capsaicin is a relatively stronger scavenger of singlet oxygen.

Fig 30. Effect of capsaicin/dihydrocapsaicin on singlet oxygen ($^1O_2$) production by riboflavin in fluorescent light.

Generation of singlet oxygen was measured by recording the decrease in the absorption of p-nitrosodimethyl aniline (pRNO) solution (0.03mM) in 10.0mM potassium phosphate buffer pH 7.8) containing 10mM Histidine as a selective acceptor of $^1O_2$. Reaction mixtures contained 1.5µg of riboflavin (Rf) and were incubated in fluorescent light for the indicated time periods.

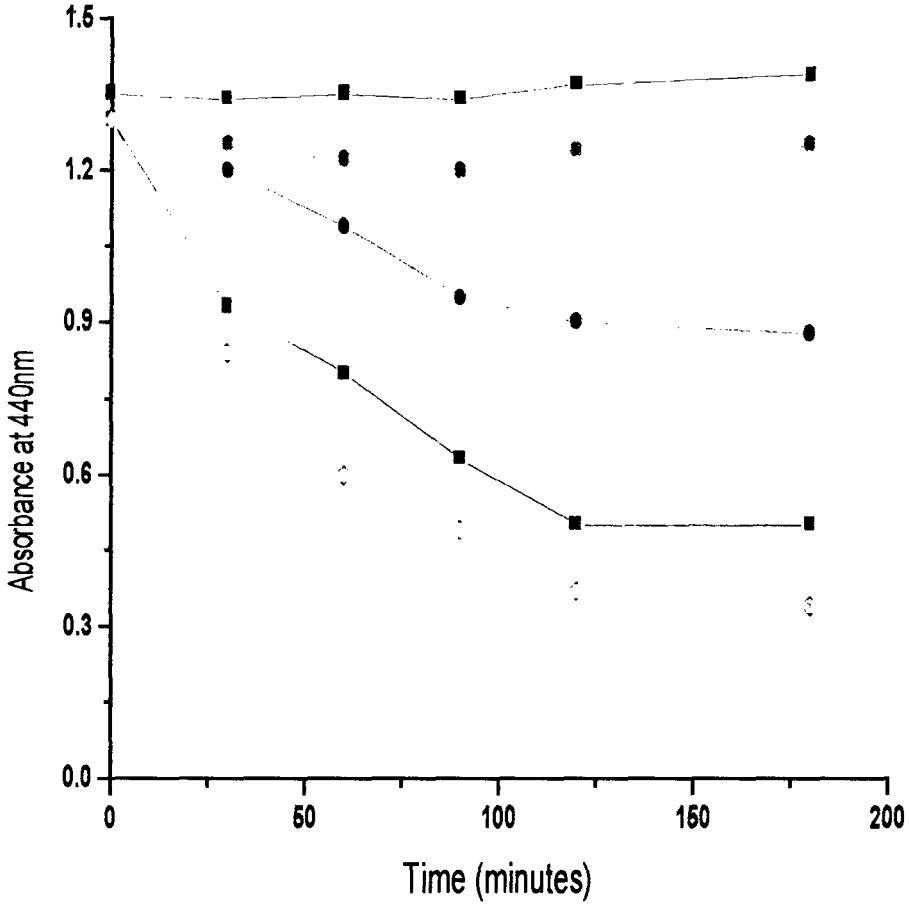
(■) – pRNO alone

(▲) – pRNO + Rf + Sodium Azide (50mM)

(●) - pRNO + Rf + Capsaicin (0.1mM)

(■) - pRNO + Rf + Dihydrocapsaicin (0.1mM)

(▼) - pRNO + Rf


1.5
1.2
0.9
0.6
0.3
0.0
Absorbance at 440nm
0
50
100
150
200
Time (minutes)

Inhibition of photosensitized riboflavin mediated degradation of plasmid DNA

Photosensitized riboflavin degrades DNA (Naseem *et al*, 1988) and in the present experimental conditions photoillumination of riboflavin leads to the conversion of supercoiled plasmid to open circular and linear forms (figure 31, lane 2). The quenchers of riboflavin triplet state namely, potassium iodide (1mM) and to a lesser extent sodium azide (1mM) inhibit the DNA degradation (lanes 3 and 4 respectively). Dihydrocapsaicin (0.1 and 0.2 mM) did not inhibit DNA degradation to the same extent (lanes 5 and 6 respectively) while capsaicin (0.1 and 0.2 mM) when present in the reaction mixture inhibits the DNA degradation (lanes 7 and 8 respectively).

Introduction of interstrand crosslinks in DNA by psoralen and inhibition by capsaicin/dihydrocapsaicin

A fifteen minute incubation of the linearized plasmid with psoralen (0.05 mM) in the presence of UV light leads to the formation of interstrand crosslinks, as revealed by the decrease in the extent of denaturation of the double stranded linear DNA to single stranded form. Figure 32 (a) shows that the presence of 0.02mM capsaicin inhibits the formation of crosslinks. However, at the same concentration dihydrocapsaicin failed to offer any inhibition. Figure 32(b) shows the effect of

Fig 31. Inhibition of photosensitized riboflavin mediated degradation of plasmid pBR322 DNA by dihydrocapsaicin/capsaicin.

DNA in 10mM Tris-HCl (pH 7.5) was incubated with 0.08mM riboflavin for 1 hour at room temperature in fluorescent light (lane 2). In addition, reaction mixtures contained: 1mM of potassium iodide and sodium azide (lane 3 and 4 respectively); 0.1 and 0.2mM of dihydrocapsaicin (lanes 5 and 6 respectively) and capsaicin (lanes 7 and 8 respectively). Lane 1; DNA alone.

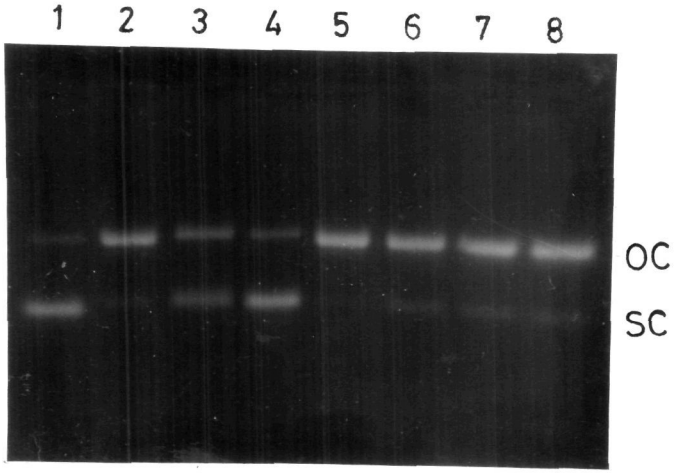

1
2
3
4
5
6
7
8
OC
SC

Fig 32. Effect of capsaicin/dihydrocapsaicin on the introduction of cross links in linearized plasmid DNA pBR322.

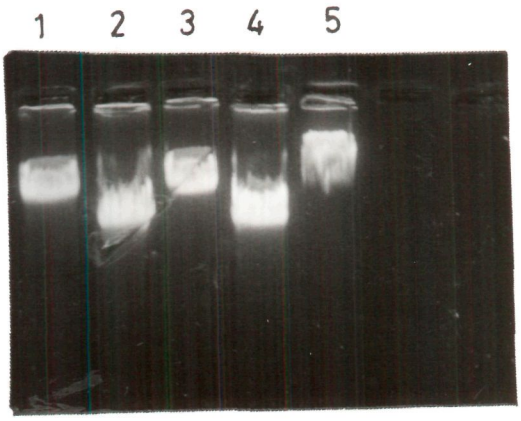
Linearized plasmid DNA (0.8μg) was incubated with 0.05mM psoralen for 15 minutes under UV light. Details of the experiment are given in "Methods".

(a) Effect of 0.1mM compound concentration

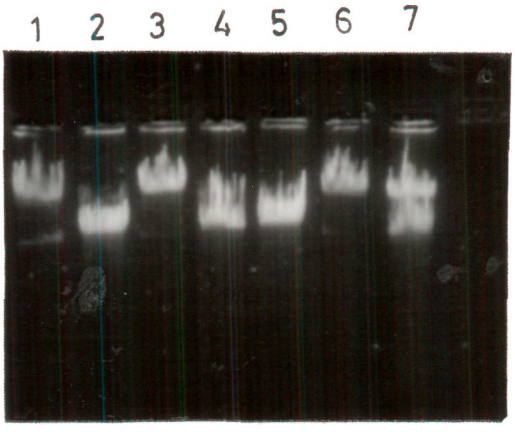
Lane 1 : DNA alone; Lane 2 : denatured DNA ; Lane 3 : DNA + psoralen ; Lane 4 : DNA + psoralen + capsaicin ; Lane 5 : DNA + psoralen + dihydrocapsaicin.

(b) Effect of 0.1 and 0.2 mM compound concentration

Lane 1 : DNA alone; Lane 2 : denatured DNA ; Lane 3 : DNA + psoralen ; Lane 4 and 5 : DNA + psoralen + 0.1 and 0.2mM capsaicin respectively ; Lanes 6 and 7 : DNA + psoralen + 0.1 and 0.2mM dihydrocapsaicin respectively.



a



b

increasing concentrations (0.1 and 0.2mM) of compounds on crosslinks. It is seen that capsaicin even at 0.1 mM offered substantial inhibition (lane 4) while at 0.2 mM almost total inhibition was seen. Dihydrocapsaicin was totally ineffective at 0.1 mM while at 0.2mM concentration, partial inhibition of crosslink formation is seen.

Fe-Ascorbate induced LDL peroxidation and inhibition by capsaicin and dihydrocapsaicin

Figure 33 shows the effect of 0.2mM capsaicin and dihydrocapsaicin on low density lipoproteins (LDL) peroxidation induced by Fenton's reagent (Fe-ascorbate). It is seen that at the end of a 150 minute incubation period, capsaicin reduces LDL peroxidation by about 47% while its structural analog dihydrocapsaicin could decrease peroxidation by only 38%. The result indicates that as in previous experiments, capsaicin is a more potent antioxidant than dihydrocapsaicin.

Inactivation of bacteriophage lambda by Capsaicin/ DHC- Cu(II).

It has been demonstrated by Husain and Hadi (1998) that oxygen free radicals generated by L-DOPA-Cu(II) in aqueous solutions cause inactivation of bacteriophage lambda. Table V assesses the inhibition of inactivation by increasing

Fig 33. Effect of capsaicin/dihydrocapsaicin on LDL peroxidation by Fenton's reagent as a function of time.

The system used for the generation of hydroxyl radical contains H_2O_2 (3.75 X 10^{-3}%), sodium ascorbate (0.125mM) and Fe(II)-EDTA (2.5μM Fe(II)-5μM EDTA). The reaction mixtures were incubated at 37^0C for different time intervals.

(◐) – Control

(●) – Capsaicin (0.2mM)

(■) – Dihydrocapsaicin (0.2mM)

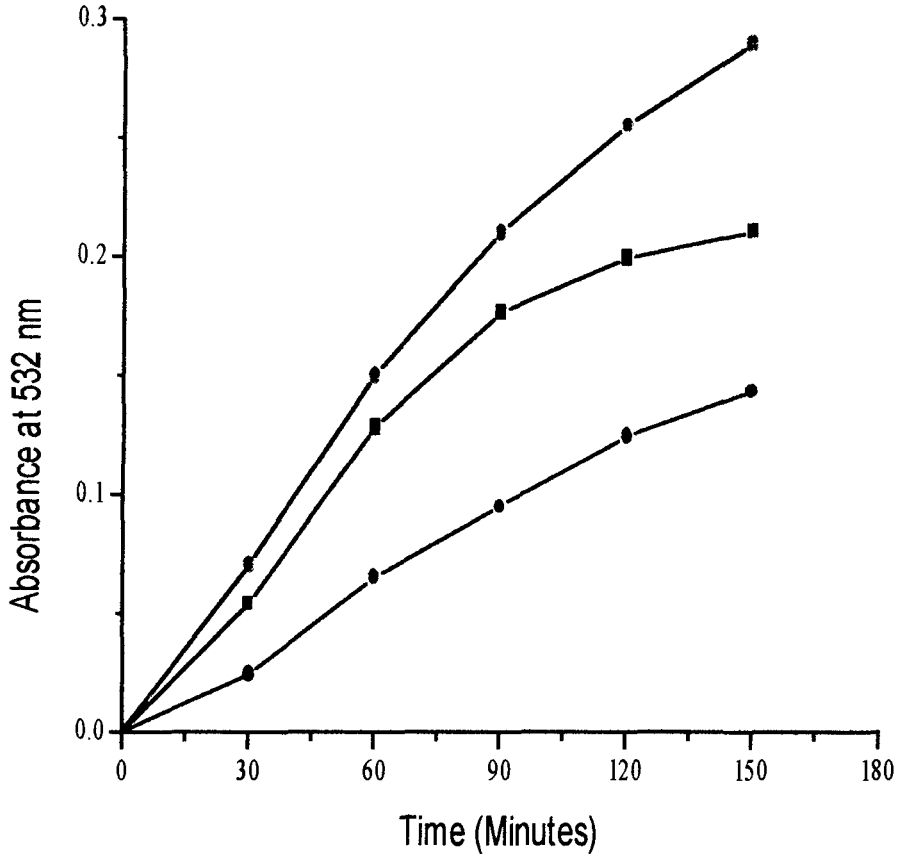

0.3
0.2
0.1
0.0
Absorbance at 532 nm
0
30
60
90
120
150
180
Time (Minutes)

Table V : Phage lambda inactivation by L-DOPA-Cu(II) and % inhibition by capsaicin(Cap)/dihydrocapsaicin(DHC)

Host	Phage treatment	PFU/ml	Survival % of control	% inhibition of inactivation
AB1157	None	1.17×10^{11}	100	-
AB1157	L-DOPA + Cu(II) (Control)	1.5×10^{10}	12.82	0
AB1157	Control + Cap (0.05mM)	2.74×10^{10}	21.74	10.23
AB1157	Control + Cap (0.1mM)	4.5×10^{10}	38.46	29.41
AB1157	Control + Cap (0.2mM)	9.5×10^{10}	81.19	78.42
AB1157	Control + DHC (0.05mM)	2.6×10^{10}	20.13	8.38
AB1157	Control + DHC (0.1mM)	3.3×10^{10}	28.20	17.64
AB1157	Control + DHC (0.2mM)	6.82×10^{10}	58.31	52.17

Concentration shown are final reaction concentrations. L-DOPA and Cu(II) were 40μM each. The recation mixtures were incubated at 37°C for 30 minutes.

PFU = Plaque Forming Units.

concentrations of capsaicin and dihydrocapsaicin. It is seen that there is a progressively dose dependent inhibition of inactivation by both the compounds. However, the inhibition by capsaicin at all concentrations is greater than that offered by dihydrocapsaicin. These results are in agreement with previous experiments showing inhibition of L-DOPA-Cu (II) mediated DNA cleavage.

In the following experiments we report that oxygen free radicals generated by capsaicin/dihydrocapsaicin-Cu(II) cause inactivation of bacteriophage lambda. The inactivating activity of capsaicin-Cu(II) and also dihydrocapsaicin-Cu(II) was determined by incubating phage lambda with compound-Cu(II) in the presence of air for 2 hours and then measuring the loss of biological activity. Figure 34 analyses the loss of survival by λvir as a function of the compound concentration. It can be seen that increasing concentrations of both capsaicin and dihydrocapsaicin results in a progressive loss of survival. However, the rate of phage inactivation is greater in the case of capsaicin. Figure 35 shows the effect of increasing time on phage inactivation by capsaicin/dihydrocapsaicin in the presence of Cu(II) as can be seen , there is a progressive decrease in phage survival with increase in time of incubation. However, the first two hours of incubation show the greatest extent of phage inactivation (upto 60%).

To determine the mechanism of phage inactivation by capsaicin/dihydrocapsaicin and Cu(II), I have studied the effects of sodium azide,

Fig 34. Effect of capsaicin(●)/dihydrocapsaicin(■) concentration on phage lambda survival in the presence of Cu(II).

The concentration of Cu(II) was 0.5mM. Incubation was at 37^0C for 2 hours. Other details of the procedure are given in "Methods".

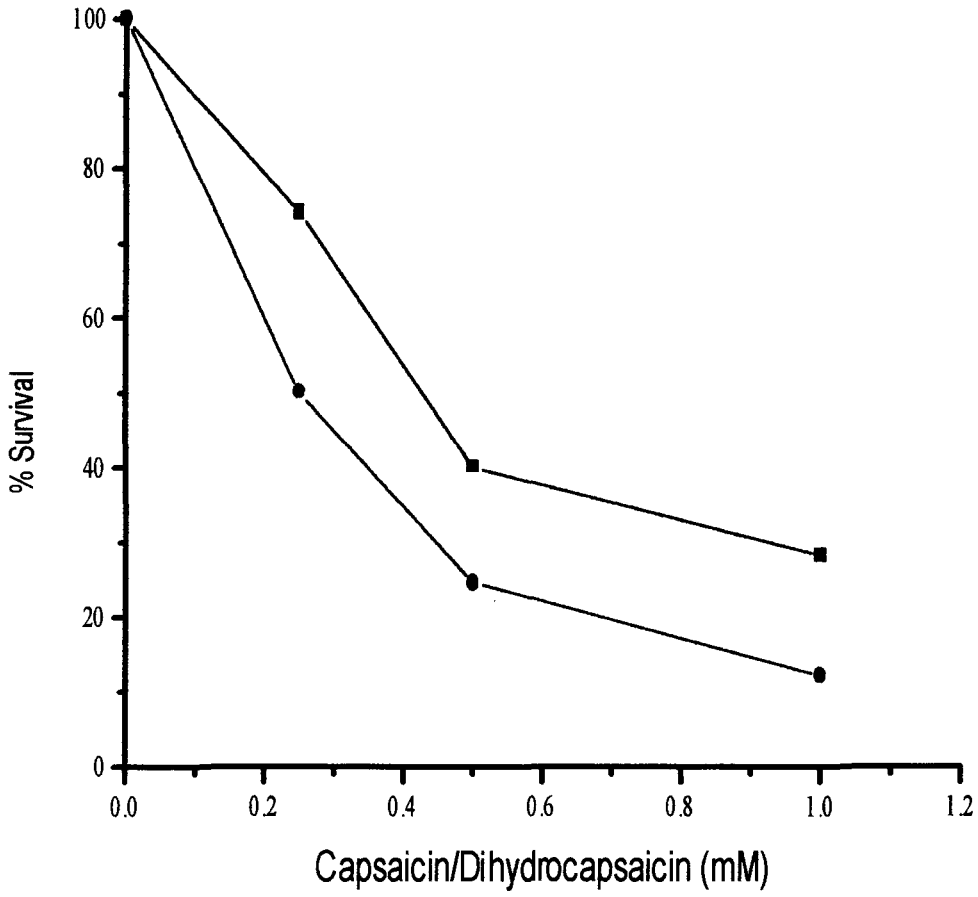
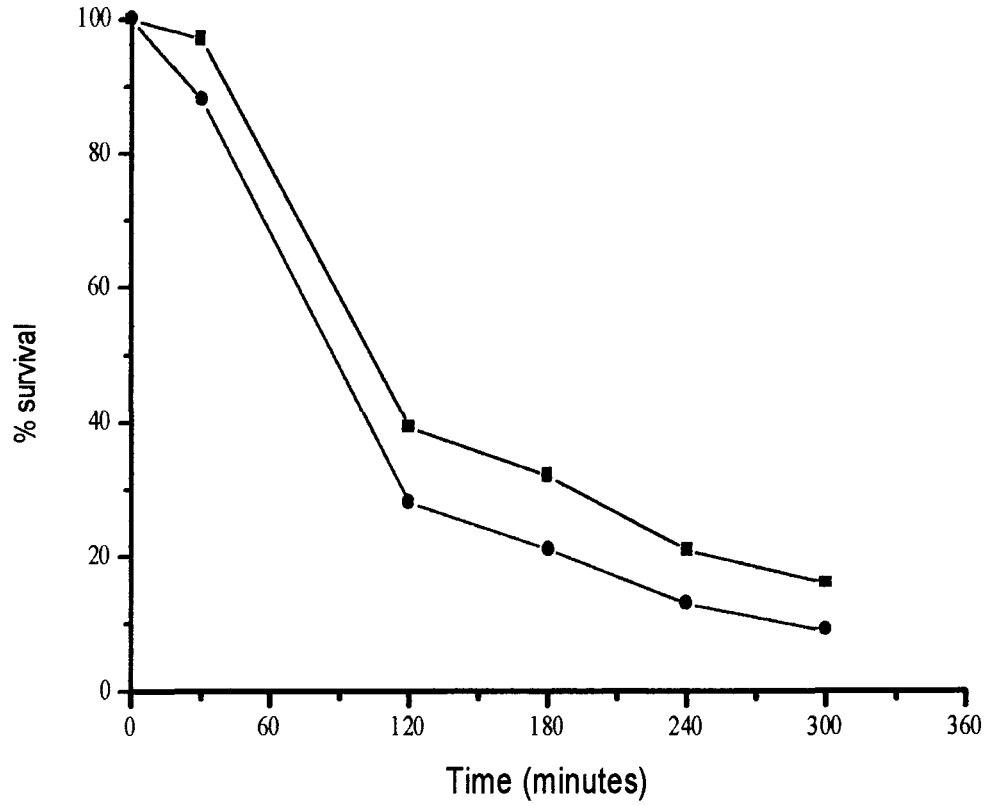

100
80
60
40
20
0
% Survival
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Capsaicin/Dihydrocapsaicin (mM)

Fig 35. Effect of time on phage lambda survival in the presence of capsaicin/dihydrocapsaicin-Cu(II).

The concentration of compounds and Cu(II) was 0.5mM. Incubation was at 37^0C. Other details of the procedure are given in "Methods".

(●) - Capsaicin

(■) - Dihydrocapsaicin


100
80
60
40
20
0
% survival
0
60
120
180
240
300
360
Time (minutes)

thiourea, catalase and superoxide dismutase (SOD) on the reaction mixtures (Table VI a and b). Inactivating activity of capsaicin/dihydrocapsaicin was reduced by prior addition of oxygen radical quenchers-sodium azide, thiourea and sodium benzoate. Catalase caused very little inhibition of inactivation while SOD was ineffective. This underlines the role of singlet oxygen and hydroxyl radicals in phage inactivation by both capsaicin-Cu(II) and dihydrocapsaicin-Cu(II).

Table VII shows the sensitivity of bacteriophage lambda to capsaicin/dihydrocapsaicin-Cu(II) and the effect of UV irradiation on the host cells. The fraction of surviving phage was 0.280 and 0.522 respectively in the case of UV untreated and treated host cells in presence of capsaicin-Cu(II). This was 0.40 and 0.505 respectively in the presence of dihydrocapsaicin-Cu(II). It may also be noted that at 0.5 mM compound concentration the fraction of phage survival in table VII (0.28 for capsaicin, 0.40 for dihydrocapsaicin) matches the degree of inactivation in figure 34 (survival 0.24 for capsaicin and 0.39 for dihydrocapsaicin). The results indicate that UV treatment of the host bacteria enhances the recovery of the phage indicating the involvement of UV inducible pathway in the repair of capsaicin/dihydrocapsaicin-Cu(II) damaged DNA.

Table VI (A) : Effect of oxygen radical scavengers on phage inactivation by Capsaicin/-Cu(II)

Host	Phage treatment	PFU/ml	Survival % of control	% inhibition of inactivation
AB1157	None	3.0×10^{11}	100	-
AB1157	Capsaicin+Cu(II) (Control)	6.9×10^{8}	23.0	0
AB1157	Control + Sod. Azide (50mM)	1.98×10^{9}	67.0	57.14
AB1157	Control + Sod. Benzoate (50mM)	1.83×10^{9}	61.0	49.35
AB1157	Control + Thiourea (50mM)	2.24×10^{9}	78.0	71.42
AB1157	Control + Catalase (0.1mg/ml)	7.5×10^{8}	29.0	7.81
AB1157	Control + SOD (0.1mg/ml)	7.0×10^{8}	24.0	1.29

Concentrations of Capsaicin and Cu(II) were 0.5mM each. The concentrations of the scavengers shown are their final reaction concentrations. Incubation was for 2 hours at 37°C followed by dilution and plating.

SOD : Superoxide dismutase.

Table VI (B) : Effect of oxygen radical scavengers on phage inactivation by Dihydrocapsaicin/-Cu(II)

Host	Phage treatment	PFU/ml	Survival % of control	% inhibition of inactivation
AB1157	None	3.0×10^{11}	100	-
AB1157	Dihydrocapsaicin+Cu(II) (Control)	9.9×10^{8}	33.0	0
AB1157	Control + Sod. Azide (50mM)	1.83×10^{9}	63.0	44.77
AB1157	Control + Sod. Benzoate (50mM)	1.71×10^{9}	57.0	35.82
AB1157	Control + Thiourea (50mM)	2.2×10^{9}	73.72	60.77
AB1157	Control + Catalase (0.1mg/ml)	9.0×10^{8}	37.0	5.90
AB1157	Control + SOD (0.1mg/ml)	6.9×10^{8}	23.0	-

Concentrations of Dihydrocapsaicin and Cu(II) were 0.5mM each. The concentrations of the scavengers shown are their final reaction concentrations. Incubation was for 2 hours at 37°C followed by dilution and plating.

SOD : Superoxide dismutase.

Table VII : Sensitivity of phage lambda to Capsaicin/Dihydrocapsaicin(DHC)-Cu(II) and the effect of UV-irradiation on the host cells

Host Strain	Host pretreatment	Phage pretreatment	PFU/ml	Survival[a]
AB1157	None	Control	$2.50 X10^9$	1
AB1157	None	Capsaicin-Cu(II)[b]	$7.00 X 10^8$	0.28
AB1157	None	DHC -Cu(II)[b]	$1.00 X 10^9$	0.40
AB1157	UV	Control	$1.78 X 10^9$	1
AB1157	UV	Capsaicin-Cu(II)[b]	$9.30 X 10^8$	0.52
AB1157	UV	DHC-Cu(II) [b]	$9.00 X 10^8$	0.50

Incubation was done for 2 hr. at 37°C followed by dilution and plating. All experiments were performed in triplicates and mean values are given.

DHC - Dihydrocapsaicin

[a] The value of survival was calculated by dividing values in the presence of Capsaicin/ Dihydrocapsaicin-Cu(II) by control values.

[b]Concentrations of compound and Cu(II) used were 0.5mM each.

Discussion

Studies in this laboratory have established that several of the proposed plant derived antioxidants such as flavonoids (Fazal *et al.*, 1990; Said Ahmad *et al.*, 1992) and tannic acid (Bhat and Hadi, 1994a; Khan and Hadi, 1998) can themselves act as prooxidants and cause DNA cleavage *in vitro* via generation of oxygen radicals, either alone or in the presence of transition metal ions such as Cu(II). In view of these reports, I have examined the prooxidant activities of capsaicin and dihydrocapsaicin (DHC) in the presence of Cu(II). Simultaneously, I have also studied and compared the antioxidant effect of these two compounds. My results support the contention that capsaicin is a **double-edged sword** as far as *in vitro* systems are concerned. I have shown that capsaicin and DHC act as both prooxidants and antioxidants and thus these compounds appear to possess properties similar to other plant derived antioxidants.

The principal conclusions of the experiments done may be stated as follows:

(1) Capsaicin and DHC in the presence of Cu(II) and molecular oxygen caused strand scission in DNA through an oxidative mechanism. The relative efficiency of cleavage was greater in the case of capsaicin.

(2) Cu(II) is reduced to Cu(I) and the latter is an essential intermediate in the DNA cleavage reaction.

(3) The proximal DNA cleaving agents are the active oxygen species such as hydroxyl radical and singlet oxygen.

(4) Antioxidant activity of capsaicin and DHC were also compared by studying the scavenging effect on hydroxyl radical and singlet oxygen generating system. Capsaicin was found to be a more effective scavenger of these radicals suggesting that the structural features of capsaicin that are important for the prooxidant action are also the ones that result in a more effective antioxidant activity.

(5) The reaction of capsaicin/dihydrocapsaicin-Cu(II) with bacteriophage lambda reduces its biological activity and this inactivation is inhibited by quenchers of active oxygen species. It thus appears that the bacteriophage inactivation is a result of strand scission in phage DNA. The results given in table VII would indicate that the inducible, error prone repair pathway on induction by UV light may also contribute to the repair of capsaicin/dihydrocapsaicin–Cu(II) mediated phage inactivation. However, this pathway is capable of operating on several types of lesions in DNA, whether produced by radiation or chemical agents (Walker, 1985).

The generation of oxygen free radicals in the proximity of DNA is well established as a cause of strand scission by various drugs. Our results indicate that capsaicin generates hydrogen peroxide and hydroxyl radicals in aqueous solutions with a greater efficiency than its structural analogue dihydrocapsaicin. We have also demonstrated the binding of DNA and Cu(II) to both capsaicin and DHC.

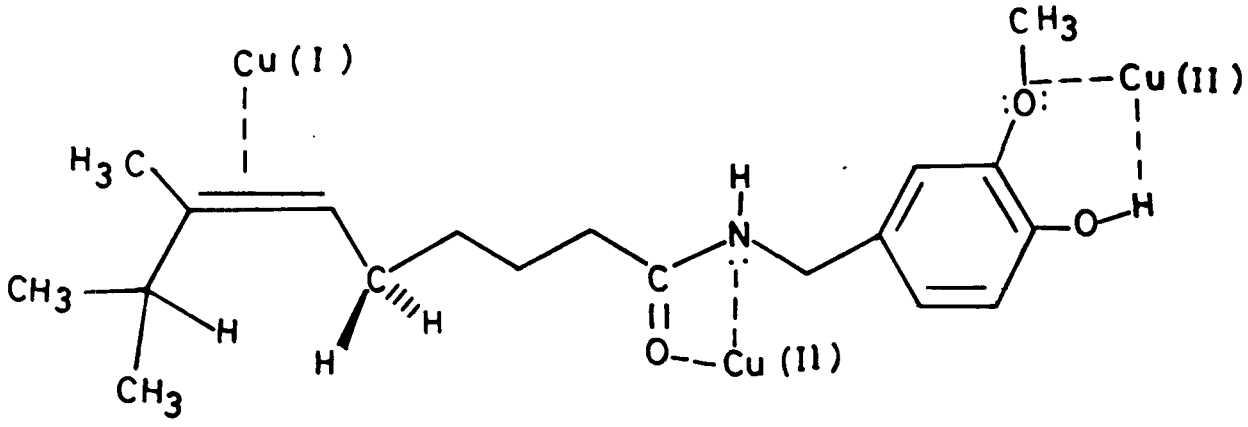
It is amply clear from the results that hydroxyl radical is a major species generated by both capsaicin and DHC, albeit to differing extents. This leads to DNA cleavage. The hydroxyl radical can arise by two alternative routes. One is the Haber-Weiss reaction (1) and the other is the Fenton's reaction (2)

$$O_2 + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^{\bullet} + O_2 \qquad (1)$$

$$H_2O_2 + Cu(II) \longrightarrow {}^{\bullet}OH + OH^{\bullet} \qquad (2)$$

These observations and our results lead us to propose a possible model for Cu(II) binding to capsaicin and DHC (Fig. 36). According to this model, both capsaicin and DHC possess a minimum of two binding sites for copper, the first of which is provided by the hydroxyl group and the methoxy group of the phenol rings, the second binding site is between the imino group and the carbonyl oxygen atom. The third site is found in the case of capsaicin and is due to the presence of the double bond on the side chain. The high electron density of this (π) bond ensures that the positively charged moieties like Cu(II) are provided another site for binding, resulting in the formation of a metal $-\pi$ complex. Thus capsaicin would have three binding sites and DHC only two. This is in concurrence with the results of the Job plots and stoichiometry which shows a stoichiometry of three for capsaicin and two for dihydrocapsaicin.

Fig 36. Proposed model for binding of copper to capsaicin.


Cu (I)
H3C
CH3
H
CH3
C
H
H
C
O
N
H
Cu (II)
CH3
O
Cu (II)
O
H

Capsaicin is known to deplete the neurotransmitter of painful impulses known as substance P from the sensory nerve terminals which provides a rationale for studying mechanisms and also for pharmacotherapy to treat some painful peripheral conditions such as rheumatoid arthritis, hepatic neuralgia, post-mastectomy pain syndrome and diabetic neuropathy (Carter, 1991; Cordell and Arujo, 1993; Dubner, 1991). Considering the frequent consumption of capsaicin as a food additive and its therapeutic application, correct assessment of any harmful effects of this compound is important from the public health point of view. The mutagenic and carcinogenic activities of capsaicin and chili extracts have been studied but the results are conflicting. It is for this reason that capsaicin has been termed by some workers as a double-edged sword (Surh and Lee, 1995). Capsaicin has been reported to have dual effects on chemically induced carcinogenesis and mutagenesis. Although a minute amount of capsaicin displays few or no deleterious effects, heavy ingestion of the compound has been associated with histopathological and biochemical changes including hepatic necrosis and ulceration (Monsereenusorn *et al.*, 1982). An epidemiological study conducted in Mexico revealed that consumers of chilli peppers were at higher risk for gastric cancer than non-consumers (Lopez-Carillo *et al.*, 1994). Further, dietary administration of capsaicin promotes duodenal tumours in Swiss albino mice (Toth and Gannett, 1992; Toth and Walker, 1984). Other studies suggest that capsaicin or chilli extracts may act as a co-carcinogen or tumour promoter (Agarwal *et al.*, 1986; Jang and Kim, 1988; Kim *et al.*, 1993). In addition, dietary capsaicin

potentates N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced gastric cancer in rats (Kim *et al.*, 1985) while no such promotional effect was observed in a study using the azoxymethane induced gastrointestinal carcinogenesis model in rats (Kang, 1992). Capsaicin is considered to be metabolized by cytochrome p-450 dependent mixed-function oxidases to reactive species (Gannett, 1990; Lee and Kumar, 1980; Miller, 1983) which may subsequently interact with target cell DNA in an irreversible manner, thereby triggering mutagenecity and malignant transformation (Surh and Lee, 1995).

On the other hand, capsaicin has been reported to ameliorate the mouse skin carcinogenesis when given simultaneously with the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Park *et al.*, 1998). It also significantly inhibited the cyclophosphamide-induced (i.p) chromosomal aberrations and DNA strand breakage (De *et al.*, 1995). The metabolism and covalent binding of benzo(α) pyrene to DNA in mouse and human keratinocytes and their suppression by capsaicin has been reported by Modley *et al.* (1986). In a series of *in vitro* studies, Teel and his associates have demonstrated that protective effects of capsaicin against metabolism, DNA binding and/or mutagenecity of some chemical carcinogens such as aflatoxin B_1 and the tobacco specific nitrosoamine, 4-(methylnitrosoamino)-1-(3-pyridal)-1-butanone (Miller *et al.*, 1993; Teel , 1991 & 1993; Zhang *et al.*, 1993). Gannett and co-workers (1990) have shown that dihydrocapsaicin, the saturated structural analogue of capsaicin is a suicidal

mechanism based inhibitor of cytochrome p450 2E1 (CYP2EI), an isoform that has an important role in the metabolic activation as well as detoxification of a wide array of chemical carcinogens of relatively small size. By analogy, capsaicin also inhibits the rodent hepatic CYP2EI activity *in vitro* (Surh *et al*, 1995). Bacterial mutagenecity of vinyl carbamate and N- nitrosodimethylamine mediated by rat hepatic S-9 and reduced nicotinamide adenine dinucleotide phosphate was also significantly reduced by capsaicin (Shlyankevich *et al.*, 1995; Surh *et al.*, 1995). Further, capsaicin has been shown to induce apoptosis in gastric cancer cell line SNU-1 by DNA fragmentation and overexpression of p53 and/or c-myc (Kim *et al.*, 1997) while both capsaicin and dihydrocapsaicin have induced apoptosis in human B cells and mouse myeloid cell lines (Wolvetang *et al.*, 1996). The ability of capsaicin to alter carcinogen metabolism provides rationale for application of this compound to chemoprevention. This effect is thought to be due to modulation of metabolism of carcinogens/mutagens and their interactions with target DNA.

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Uric acid inhibits L-DOPA-CU(II) mediated DNA cleavage

S. Singh, S. Farhan Asad, S.M. Hadi*

Department of Biochemistry, Faculty of Life Sciences, A.M.U., Aligarh, 202 002, India

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Abstract

It has been proposed that considerable DNA damage may be caused by endogenous metabolites produced during the body's normal metabolic processes. We have previously shown that L-DOPA, in the presence of Cu(II) leads to oxidative DNA breakage in vitro. Uric acid is considered to be a naturally occuring antioxidant and is present in plasma at a relatively high concentration. In this paper we report that uric acid inhibits L-DOPA-Cu(II) mediated DNA cleavage at concentrations similar to or lower than those found in plasma. Xanthine, which is the structural analogue of uric acid is a more potent inhibitor of the reaction. Uric acid was also shown to directly quench the generation of hydroxyl radicals by L-DOPA-Cu(II). The results have been discussed in relation to the putative protective role of uric acid against endogenous DNA damage by oxygen radicals. © 1998 Published by Elsevier Science Ireland Ltd. All rights reserved

Keywords: L-DOPA; Copper; Endogenous DNA damage; Uric acid; Antioxidant; Oxygen radicals

It has been proposed that considerable DNA damage may be caused by endogenous metabolites produced during the body's normal metabolic processes. For example, it has been shown that malondialdehyde which is the ubiquitous product of lipid peroxidation and icosanoid metabolism reacts with cellular DNA to form a propanodeoxyguanosine adduct [2,4]. Evidence indicates that the adduct exists at significant levels in the hepatic DNA of rats and humans and is an efficient premutagenic lesion in *E. coli* [2,5]. Dopamine, formed by the decarboxylation of L-DOPA [11], condenses with acetaldehyde, a product of ethanol metabolism, to generate 1-Methyl-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisoquinoline (Salsolinol) [7]. Salsolinol is considered to be involved in the etiology of Parkinson's and Huntington's diseases and has been detected in the cerebrospinal fluid of Parkinsonian patients [13]. Thus, dopamine can be considered a precursor of an endogenous neurotoxin. We have shown that L-DOPA causes DNA breakage in vitro in the presence of transition metals such as Cu(II) and that the reaction is catalysed by the formation of reactive oxygen species [10]. Copper has been shown to be a normal component of chromatin and is available for inter-nucleosomal DNA fragmentation in isolated nuclei [3]. Halliwell et al. [20] have proposed that copper ions released in the presence of L-DOPA and its metabolites may be an important mechanism of neurotoxicity in several neurological disorders [20]. A number of molecules present in human extracellular fluids are considered to have antioxidant function [9]. These include ascorbic acid and uric acid. Uric acid is present in human plasma at a relatively high concentration (up to 0.6 mM) and is capable of scavenging hydroxyl radical, lipid hydroperoxides, singlet oxygen and oxo-heme oxidants [1]. In this paper, we report that uric acid inhibits L-DOPA-Cu(II) mediated DNA cleavage and quenches the hydroxyl radical production by the same system at concentrations similar or lower to those found in plasma.

Calf thymus DNA (sodium salt; average MW: 1×10^6) and S_1 nuclease were from Sigma (St. Louis, MO). L-DOPA was obtained from Fluka (Switzerland). Supercoiled plasmid pBR322 DNA was prepared according to standard methods [12].All other chemicals were of analytical grade.

Formation of single strand breaks by L-DOPA-Cu(II) and their inhibition by uric acid was assayed by the extent of generation of S_1 nuclease sensitive sites in Calf thymus

* Corresponding author. Tel.: +91 571 400741; fax: +91 571 400466; e-mail: smhadi@mailcity.com

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DNA [16]. Induction of DNA strand breaks and the inhibition of the reaction by uric acid and xanthine was further assessed by the decrease in superhelicity and linearization of supercoiled plasmid pBR322 DNA [10]. Reaction mixtures (30 μl) contained 10 mM Tris–HCl (pH 7.5), 0.50 μg plasmid DNA and varying concentrations of uric acid. Incubation at room temperature was for 20 min. To compare the effects of uric acid and other known hydroxyl radical scavengers on the generation of hydroxyl radicals by the L-DOPA-Cu(II) system, the method of Quinlan and Gutteridge [15] was followed. The reaction mixtures (0.5 ml) contained 2 mM deoxyribose, 50 μM L-DOPA and indicated concentrations of uric acid, mannitol and thiourea.

We have previously shown that L-DOPA in the presence of Cu(II) generates S_1 nuclease sensitive sites in calf thymus DNA [10]. The reaction records the proportion of DNA converted to acid soluble nucleotides by the nuclease. Fig. 1 gives the kinetics of such an experiment in the absence and presence of 300 μM uric acid. At the end of a 4 h incubation period, a decrease of about 30% in the amount of acid soluble material produced is observed. The percent inhibition caused by uric acid after 4 h of incubation was found to be significant ($P < 0.001$).

The inhibitory effect of uric acid was also examined on the cleavage of supercoiled plasmid DNA as the relaxation of such a molecule is a sensitive test for just one nick per molecule. Fig. 2a shows the effect of three different concentrations of uric acid on the conversion of supercoiled pBR322 DNA to open circular and linear forms. It is seen that the formation of linears is inhibited by 200 μM uric acid and completely inhibited at a 400 μM concentration (lane 4). As xanthine is the structural analogue and the metabolic precursor of uric acid, it was also of interest to observe its effect on single strand breakage in the plasmid by the L-DOPA-Cu(II) system. The results indicate that xanthine is a better inhibitor of the reaction as the full conversion of molecules to the relaxed form is prevented at even 100 μM concentration and the formation of linears is not seen at all.

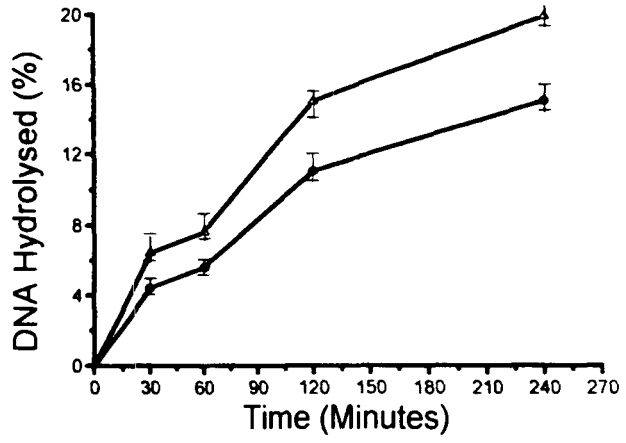

Fig. 1. Kinetics of degradation of calf thymus DNA by L-DOPA-Cu(II) in the presence (●) and absence (Δ) of uric acid (300 μM). The concentrations of L-DOPA and Cu(II) were 100 μM each.

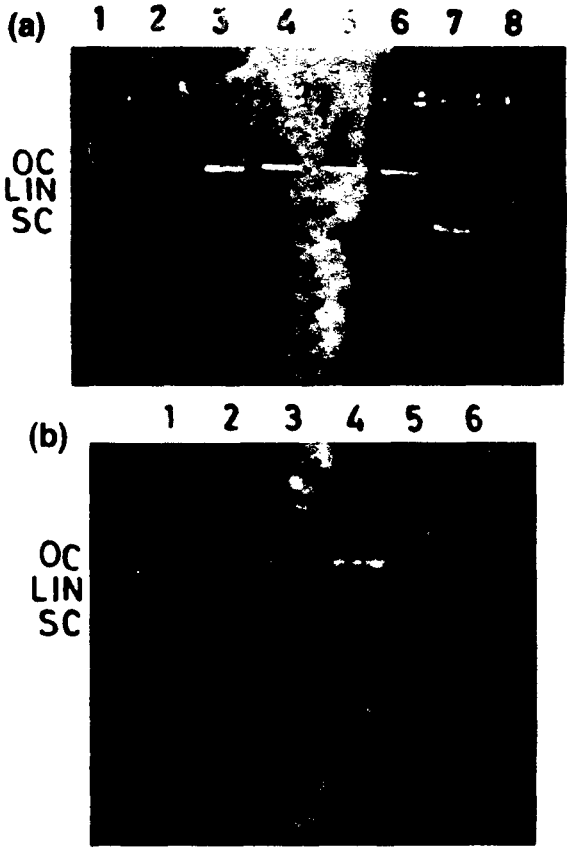

Fig. 2. Effects of various antioxidants on cleavage of supercoiled plasmid DNA by L-DOPA and Cu(II) (50 μM of each). (a) Effect of different concentrations of uric acid and xanthine. Lanes: 1, DNA alone; 2, L-DOPA + Cu(II); 3–5, 100, 200 and 400 μM uric acid, respectively; 6–8, 100, 200 and 400 μM xanthine, respectively. (b) Effect of uric acid, xanthine, mannitol and thiourea. Lanes: 1, DNA alone; 2, L-DOPA + Cu(II); lanes 3–6, uric acid, xanthine, mannitol and thiourea, respectively (200 μM each).

The oxidative DNA breakage by L-DOPA-Cu(II) system was earlier shown to involve the hydroxyl radical as the proximal DNA cleavage agent [10]. We have, therefore, compared the inhibitory effect of uric acid and xanthine with two known scavengers of the hydroxyl radical, namely mannitol and thiourea. Results given in Fig. 2b confirm that xanthine exhibits a greater inhibitory effect than uric acid (lanes 3 and 4). However, both mannitol and thiourea (lanes 5 and 6) do not show any inhibition of the cleavage at the concentrations (200 μM) used for uric acid and xanthine. It may be noted that the concentrations of mannitol and thiourea required for observing in vitro protection against DNA cleavage by L-DOPA-Cu(II) is 50 mM [10]. This explains the effect of these two scavengers in the above experiment.

We directly tested the effect of uric acid on the generation of hydroxyl radicals by L-DOPA-Cu(II). The assay used involves the reaction of hydroxyl radicals with deoxyribose leading to a free radical intermediate that decomposes to form an aldehyde which in turn gives an adduct with TBA. The effect of two concentrations of uric acid on the kinetics of hydroxyl radical generation was studied and a

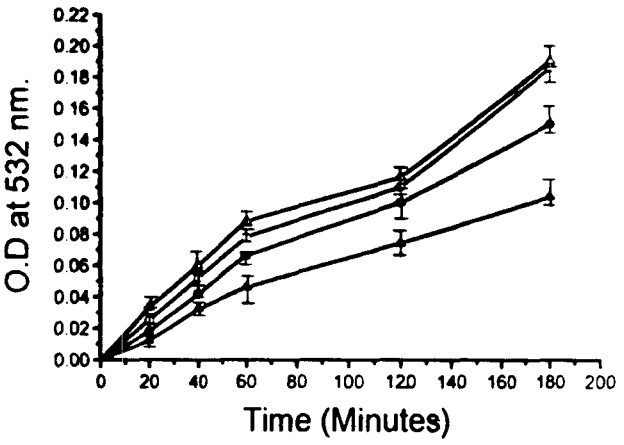

Fig. 3. Comparison of the quenching effect of uric acid (▲), mannitol (○) and thiourea (●) on hydroxyl radical generation by L-DOPA and Cu(II). The concentration of L-DOPA and Cu(II) was 50 μM and 100 μM, respectively (△), and that of quenchers was 25 μM each.

dose-response relationship was observed as the inhibitory effect of 50 μM uric acid was greater than 25 μM uric acid (results not shown). The quenching effect of uric acid was also compared with thiourea and mannitol. In the presence of uric acid, the rate of formation of hydroxyl radicals was reduced to about 50% at the end of a three hour incubation period. At the same concentration (25 μM), mannitol did not show any effect whereas thiourea was inhibitory to the extent of about 20% (Fig. 3). The inhibitory effects of mannitol, thiourea and uric acid at 180 min incubation were significant with $P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively. Further, the inhibition by uric acid with respect to mannitol and thiourea was also found to be significant ($P < 0.001$).

The normal physiological concentration of uric acid in plasma is 3–9 mg/dl or 0.2–0.6 mM [14]. Under certain diseased conditions such as hyperuricemia, gout and arthritis, the uric acid concentration is increased two to four times than normal. In the present studies, the inhibitory concentrations of uric acid for L-DOPA-Cu(II) mediated DNA cleavage is 0.2–0.4 mM. The concentration required for direct scavenging of L-DOPA-Cu(II) generated hydroxyl radicals is even lower (25–50 μM). Thus, these studies indicate that at the physiological concentrations found in blood, uric acid is capable of exerting a scavenging effect on L-DOPA-Cu(II) generated DNA damaging oxygen radicals. Further, the studies support the putative antioxidant role of uric acid in higher primates.

Having stated the above, it is to be recognised that it is not established whether uric acid is present in the cell nucleus. Although it is hydrophilic in nature, it is conceivable that as a complex with copper, uric acid is capable of traversing the cell or nuclear membrane. Uric acid binds metal ions such as copper and iron and therefore mobilizes free or loosely bound copper and iron [6]. In addition to chromatin, normal serum contains upto 8 μM loosely bound copper [3,17]. Loosely bound copper is defined by Gutteridge as that copper which is available for binding to the chelating agent 1, 10-phenanthroline [8]. It is possible that such loosely bound copper is also available for binding to uric acid. Several of the known antioxidants such as flavonoids in plants and ascorbate in animals are known to generate reactive oxygen species in the presence of transition metal ions and lead to DNA cleavage [18]. Similarly, we have shown that uric acid in the presence of Cu(II) is capable of causing strand scission in DNA and that this reaction is associated with the generation of hydroxyl radicals [19]. However, the concentration of uric acid required for any significant DNA cleavage is 1 mM. Irrespective of the physiological significance of our results it is suggested that whereas potential endogenous DNA damaging reactions may occur, the animal system also provides for preventive mechanisms against such damage.

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